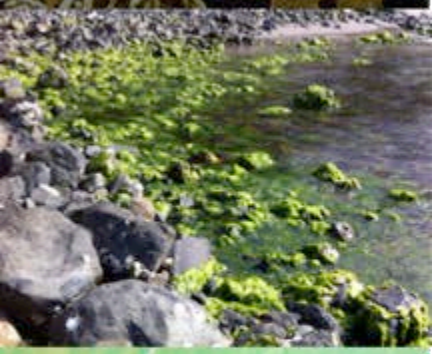

USING MACROPHYTES TO IMPROVE THE UNDERSTANDING AND MANAGEMENT OF METAL CONTAMINATED ESTUARIES

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Submitted in fulfilment of the requirements for the Doctor of Philosophy

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STATEMENTS AND DECLARATIONS

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The candidate was the primary author who conceived the idea, conducted experiment, analysed data and built manuscript (60%). Catriona Hurd provides advice on analytical technique, data analysis and manuscript preparation (10%). Ruth Eriksen provide advice

in the manuscript (5%), Carmen Simioni (5%) and Eder Schmidt (5%) assist for cytology laboratory process and data analysis, and Zenilda Bouzon provides funding and expertise for microscopy analysis (5%). Catriona Macleod as primary supervisor assisted providing advice and funding, manuscript preparation, and framing concept (10%). The Derwent Estuary Program (DEP) provided part of the environmental data.

- **Paper 2 (Chapter 4)**

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GENERAL ABSTRACT

Estuarine and coastal environments support a broad range of anthropogenic activities and many of these can have adverse interactions. Metal pollution is a particular concern because metals persist in the environment and can have a toxic effect on living organisms. Macrophyte species have been shown to be effective for monitoring and removing pollutants such as inorganic compounds and nitrogen, but their potential for removing heavy metals is still poorly understand. Understanding the ecophysiological responses of macrophyte species, in relation to metal pollution, is fundamental to developing effective monitoring and management strategies, and also to identifying potential bioremediation applications. This investigation focused on ascertaining which macrophyte species would be the best candidates for i) biomonitoring and ii) bioremediation potential of metal pollution in a heavily metal polluted estuarine/ coastal environment, the Derwent Estuary, in Hobart, Tasmania, Australia.

The potential use of local macrophyte species as biomonitors of metal pollution was assessed firstly through a broad-scale survey looking at macrophyte distribution along a contamination gradient in the Derwent. Twelve species were evaluated including three species of seagrasses and nine macroalga. The macroalga *Ulva australis* appeared to be the best candidate for a local bioindicator, because of its widespread distribution and capacity to accumulate high metal content (Arsenic (As), Copper (Cu), Lead (Pb) and Zinc (Zn)) relative to the ambient concentrations in the Derwent Estuary.

The next stage of this study reviewed the distribution of *U. australis* throughout the estuary over 3 years (2013-2015) to determine how reliable it might be as a biomonitor

over time. The relative concentrations of As, Cu, Pb and Zn were evaluated, with only Pb and Zn showing a clear spatial trends of accumulation in the tissue. This strong spatial gradient in metal content in *U. australis* appears to be most strongly related to the metal concentrations in surface water. Zinc was by far the most significant metal contaminant, and showed clear seasonal differences in levels being greater in spring and summer compared with other seasons. As a result, the next level of investigation, looked at metal uptake mechanisms and the potential to use *U. australis* for remediation focused on Zn.

In order to use macrophytes most effectively for biomonitoring or bioremediation it is important to understand how plants might respond under different levels of contamination; it is especially important to understand at what concentrations metal contamination might actually limit physiological function and/ or uptake response. However, in order to study the effect of heavy metals on the ultrastructure of *U. australis*, a new fixation protocol had to be developed to ensure the quality of specimen preservation for analysis using transmission electron microscopy (TEM). Five protocols were compared, and the most successful (2.5% glutaraldehyde, 0.05 M sodium cacodylate buffer and 2% paraformaldehyde) was applied in subsequent analyses.

The physiological and cytochemical response of *U. australis* was assessed against three Zn concentrations (25 $\mu g \cdot L^{-1}$, 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$) for seven days. There were marked utrastructural changes in *U. australis* associated with increasing Zn concentrations such as the incorporation of electron-dense bodies, cytoplasm retraction and compression of cellulose fibrils. However, the photosynthetic performance (Fv/Fm and ETRmax) and photosynthetic pigments (Chl *a*, Chl *b*, and carotenoids) were not affected by Zn concentrations. This suggests that despite the substantial cellular changes,

the plant physiology was not adversely affected, indicating that *U. australis* would appear to be adaptable to high levels of Zn and as a result has great potential to be used as a bioindicator of metal pollution.

The assessment of *U. australis* as a potential biomonitoring tool and for removing metals was further evaluated via *in situ* transplantation. Ultrastructural changes, such as the incorporation of electron-dense bodies in the cell wall and vacuoles, indicated metal accumulation at highly polluted sites. However, as in the laboratory experiment (Chapter 5), after 12 days of field deployment neither photosynthetic performance (Fv/Fm and ETRmax) nor photosynthetic pigments (Chl *a* and Chl *b*) were affected. Longer deployments (45 days), confirmed the metal bioaccumulation capacity of *U. australis* with further evidence of increases in metal content in thalli, which again suggests that this species could be a useful bioremediation tool.

In conclusion, this study suggests that *U. australis* could be a valuable tool for both monitoring and management of heavy metal contamination in Australian estuaries and coast. This study also provides a new insight into the ecophysiological response of *U. australis* to metal contamination (both *in vitro* and *in situ*), particularly zinc, and describes a new and improved technique for fixation that will lead to better definition of cellular ultrastructure in this species. It is hoped that this information will be used to support and enhance existing management and monitoring strategies in metal contaminated environments.

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CHAPTER 1

General Introduction

1.1 Estuarine ecosystems and metal contamination

Estuaries are unique environments in which the mixing of freshwater with seawater creates a naturally productive ecosystem (Potter et al. 2010; Kennish 2002), which is often important ecologically as feeding and nursery areas for coastal and estuarine species. Humans have historically concentrated around estuaries, and this has resulted in these water bodies being vulnerable to anthropogenic impacts (Guisti 2001). Some of the more common humans impacts around estuaries are associated with maritime transportation, industrial development and ports (McLusky and Elliott 2004), all of which can alter or modify estuarine/coastal habitats, communities and add a range of contaminants (Kennish 1992). Whilst there are many possible contaminants or pollutants in estuaries, nutrients, hydrocarbons, and metals are of particular concern for both human health and environmental management.

The Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP) described marine pollution as "the introduction by man, directly or indirectly, of substances (or energy) into the marine environment, resulting in deleterious effects such as harm to living resources, hazards to human health, hindrance to marine activities including fisheries, impairment of quality for use of seawater and reduction of amenities" (Kennish 1996). As there is no formal definition of estuarine pollution, this definition of marine pollution will be used for both estuarine and coastal pollution.

- **Metals in estuarine systems**

Human usage of estuarine and coastal areas (including the adjacent catchments) has resulted in increasing pollutants levels from; agriculture, recreational activities, fishing, maritime transport, mining, aquaculture, and domestic and industrial wastes. All of these uses can be a source of metal contamination (Kennish 1992). Figure 1.1 (above line) summarises the key potential sources of metals in aquatic systems. Whilst some metals are essential for metabolism in low concentrations, "transitional" or "trace" metals, these same metals can be lethal at high concentrations e.g. copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) or cobalt (Co). There are other metals, that are not essential for metabolism, the "metalloids", but these are toxic even at low concentrations (e.g. cadmium (Cd), lead (Pb), mercury (Hg) or arsenic (As) (Kennish 1996). Finally, "heavy metals" are metals with relatively high density, which includes trace metals and metalloids (Tchounwou et al. 2012). Many biologists use these terms without distinction or just refer to metals in general, without acknowledging these toxicity differences. In this study, I was investigating the effects of "heavy metals" i.e. those metals that can cause severe damage to a diverse range of organisms at low concentrations. Heavy metals can be a particular problem in estuarine systems, as they persist in the environment (Phillips and Rainbow 1994) and as such have been known to cause acute toxicity or deleterious effects in a range of living organisms (Hurd et al. 2014). Macroalgal communities are amongst those that have been found to be adversely affected by metal contamination, with both direct effects (e.g. reduction in macroalgal diversity) observed with metal contamination from sewage discharge (Liu et al. 2007), and indirect effects (e.g. reduce the number and biomass of macro-invertebrates associated with *Lessonia trabeculata* in sub-tidal communities) as a result of heavy metals from mining activities (Vasquez et al. 1999).

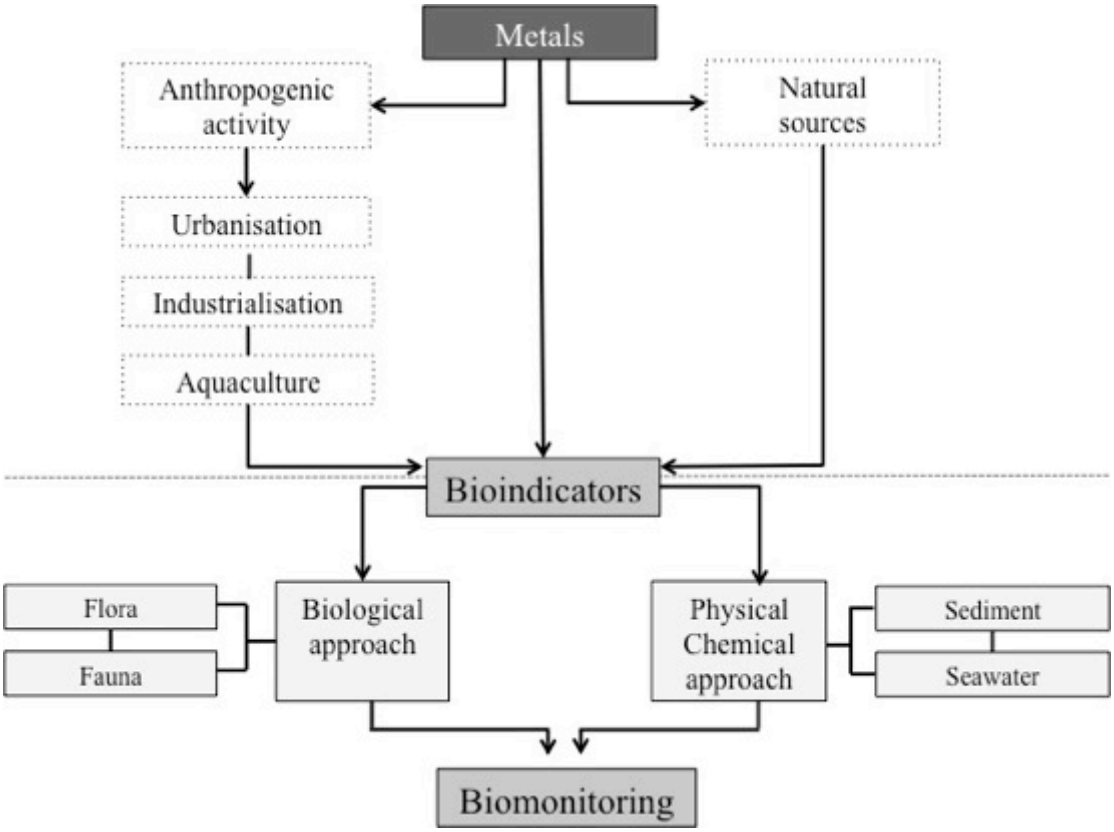

Figure 1.1. Above the dotted line, summarises the key sources of metal in aquatic environments (including both natural and anthropogenic/ pollutant sources). The potential mechanism that might be used to inform/ understand metal pollution dynamics in aquatic systems are shown below the dotted line.

- **Worldwide pollution in estuaries**

Heavy metal pollution is a global phenomenon and has been demonstrated in estuaries worldwide (Table 1.1). There are some very good examples and keystone studies showing both ecological and humans impacts of metal pollution. In the northern hemisphere, San Francisco Bay has a long history of gold mining activities and mercury pollution, and there have been many studies of the ecological effects and possible management and remediation approaches (Gehrke et al. 2011; Hornberger et al. 1999). In China, the growing population has caused an increase in metal inputs into aquatic environments over the last two decades, and as a result the risk to humans health has also increased; metal levels in bivalves have frequently been shown to represent a risk to humans associated

with seafood consumption (Pan and Wang 2012). In Tasmania (Australia), the Derwent Estuary has been shown to be amongst one of the most contaminated estuaries globally since the 1970s (Coughanowr et al. 2015). As a result, a number of studies have been undertaken to define the ecological risk (Verdouw et al. 2010; Jones et al. 2013; Macleod and Helidoniotis 2005) and to support management and remediation.

Table 1.1. Comparison of global metal concentrations (ppm) in sediments (<63 µm) from aquatic environments affected by metal pollution.

Reference	Country	Area	Source	Cu	Hg	Zn	As	Cd	Pb
Dean et al. (2007)	Scotland	Loch Craignish	Fish cages	805	-	921	-	3.5	-
Malea and Haritonidis (1999a)	Greece	Thermaikos Gulf	Sewage	6.1	-	42.2	-	1.1	130
Bryan and Langston (1992)	UK	Restronguet Creek		-	0.46	**2 821**	**1 740**	1.53	341
	UK	Gannel Estuary	Mining	-	0.08	940	174	1.35	**2 753**
	UK	Mersey			3.01	379	41.6	0.7	124
Attrill and Thomes (1995)	UK	Thames Estuary		348	5.7	**1 050**	45	9.8	**1 634**
Gehrke et al. (2011)	USA	San Francisco Bay	Mining	-	1.52	-	-	-	-
Guisti (2001)	UK	Coastal area	Mining	230*	-	**2 230***	-	11.2*	**1 137**
Whitehead et al. (2010)	Australia	Derwent Estuary	Industrial	**1 490**	**130**	**59 000**	**1 400**	477	**8 120**

* Sediment grain size <180 µm

- **Australian regulation for management**

The Australian and New Zealand Environment and Conservation Council (ANZECC) are responsible for providing management guidelines to protect environmental values and water quality, and are used to manage the potential harmful effects of contaminant in marine- and fresh- waters. The guidelines provide limits for pollutants that reflect the levels above which there is a risk of adverse effect in waters, sediments and biota (ANZECC 2000). Although ANZECC has increased the number of living organisms on their guidelines (i.e. fish, mussel and oysters), there are still no risk limits proposed for contaminants in macrophytes (ANZECC 2000). An understanding of metal toxicity in macrophytes, including data on how such plants take up and process metals, would provide the information needed to better assess the potential toxicity of macrophytes in highly metal contaminated areas and would assist in the future management of contaminated systems.

- **The Derwent Estuary as a case study**

The Derwent Estuary has many unique and important ecological features, and is home to iconic Australian species such as black swans (*Cygnus atratus)*, seadragons (*Pyllopteryx taeniolatus*) and spotted handfish (*Brachionichthys hirsutus*) (Whitehead et al. 2010). The Derwent is also economically important for recreational fishing, marine transport and boating (Whitehead et al. 2010). However, the Derwent estuary is highly contaminated with metals. In the 1970's Bloom and Ayling (1977) found high levels of metals in sediment, water, fish, and shellfish, which include Zn, Hg, As, Cd, Cu and Pb, primarily derived from metallurgical waste. The major source of these metals was industrialization, with Zn being the main contaminant in the estuary, principally as a result of spillage and local scale/ groundwater contamination from the Nystar Hobart smelter. Nystar Hobart is

one of the largest Zn producers in the world, located on the banks of the middle-upper estuary (Coughanowr et al. 2015). Lead is also introduced into the Derwent as a secondary product in the Zn leaching process, and the levels of Pb in the Derwent sediments surface layers ranged from 50 - 220 $mg \cdot g^{-1}$ (Whitehead et al. 2010). Given the ecological relevance of the Derwent Estuary and the scale of metal pollution in the estuary, this system provides a particularly interesting case study for assessing metal pollution.

In response to concerns over heavy metals in the Derwent, the Derwent Estuary Program (DEP) was created in 1999 as a voluntary partnership between the Local and State governments, the community, and various industries. The purpose of the partnership is to improve environmental conditions in the estuary, in particular working with the industry and local government to reduce pollution, improve water quality and preserve ecological habitats and unique species, but the program also undertakes regular monitoring (Whitehead et al. 2010). To date DEP research has been focused on determining levels of contamination in sediments, water, fish and invertebrates, and in all cases there are regions within the estuary where the level of metals is above the trigger levels recommended by the ANZECC guidelines (Coughanowr et al. 2015). However, so far macrophytes have not been included in any specific research on management actions.

1.2 Monitoring and Remediation

- **Monitoring metals from aquatic systems**

For assessing levels of metal in aquatic environments, the most common approach is to chemically analyse the levels of metals in sediments, water column, or living organisms. Chemical analysis of levels in the water column only give a ‘snap-shot’ of the amount of

metals in the system, however, analysis of the levels in biota/ living organisms provide a more integrated 'long-term' measure of the metal inputs and the likely effects of those inputs in the system. Metals are taken up and accumulated by many organisms (e.g. invertebrates and seaweeds) (Kennish 1996) in some cases consistent with the background loading, but in some cases at levels above background. Consequently, obtaining the metal levels for key species may give a better indication of the potential impact on the ecosystem. Organisms that are used for metal detection are termed 'bioindicators', 'biomonitors' or 'biomarkers' (Zauke et al. 1998; Kennish 1996). Indicator organisms often include mussels, oysters, barnacles, crustaceans and seaweeds (Rainbow and Phillips 1993; Rainbow 2006). Bioindicators can accumulate metals into their systems and this 'bioaccumulation' capacity can then be used to assess environmental contamination (Zauke et al. 1998; Kennish 1996). The environmental assessment of contamination using bioindicators is known as 'biomonitoring' (Zhou et al. 2008). Figure 1.1 (below line), summaries biological and chemical approaches used to inform/ understanding metal pollution dynamics in aquatic systems.

• Pollutants remediation

A number of different techniques have been proposed for removing heavy metals from water (i.e. adsorption, ion exchange and membrane filtration), but the selection of the appropriate technique depends on the initial metal concentration, the cost and the concern of environmental impacts (Fu and Wang 2011). Bioremediation is a process that relies on living organisms to help clean up polluted environments by taking up chemical contaminants (Kumar et al. 2011; Juwarkar et al. 2010; Vidali 2001). Bioremediation relies on 'Biosorption', which is the mechanism that describes both the "passive" (uptake) and "active" (bioaccumulation) accumulation of metals, and involves live or dead

biomass (Oliveira et al. 2011; Volesky and Holan 1995). There is evidence of seaweed biosorption capacity, but there is still knowledge missing about the potential of seaweed for bioremediation of metal pollution in aquatic systems.

- **Macrophytes in estuarine systems**

In estuarine systems, the key benthic primary producers are seagrasses and seaweeds. Seagrasses are an ecological group of angiosperms adapted to saline habitats (den Hartog and Kuo 2006), and they play an important role in estuarine ecosystems, providing habitat, shelter and food for other organisms (e.g. fish), improving water quality and providing a substratum for epiphytic organisms (Larkum et al. 2006). A number of studies have suggested that seagrass populations are declining in estuarine systems, as a result of their susceptibility to anthropogenic impacts (Ralph et al. 2006; Govindasamy et al. 2011).

Seaweeds include green (Chlorophyta), red (Rhodophyta) and brown (Ochrophyta) macroalgae (Hurd et al. 2014), and have many similar ecological functions to seagrass. They form the basis of the marine food chain and provide shelter for a broad range of organisms, they are also harvested as a food for humans e.g. wakame and nori, and are collected for use in the hydrocolloids industries (agar, alginate and carrageenan) (McHugh 2003) and as fertilizer and biofuels (McHugh 2003; Souza et al. 2012; Almela et al. 2002). Consequently, it is important to understand the impacts of metals and the extent to which metals might be accumulated in these primary producers.

- **Macrophytes as environmental management tool**

It is well known that seaweeds can accumulate and incorporate metals from the sediment, the water column and also suspended particles (Kennish 1996). The polysaccharides and proteins present in seaweeds have a high affinity for heavy metals, effectively acting as a biofilter to uptake metals (Ródenas de la Rocha et al. 2009; Robledo 1993). As a result of this capacity to accumulate and incorporate metals, a number of previous studies have shown that seaweeds can be excellent bioindicators of metal exposure (Zhou et al. 2008; Jayasekera and Rossbach 1996; Kennish 1996). Whether this is the case for the macrophytes in the Derwent has not yet been investigated.

With the development of aquaculture worldwide, algae have been increasingly used as a by-product or remediation tool to "utilise" or "clean up" aquaculture nutrients associated with intensive modern finfish aquaculture (Chung et al. 2002); this combines fish and seaweed aquaculture as an effective environmental management tool for removal of excess nutrients (Chopin 2011; Buschmann et al. 2009; Troell et al. 1997) in a process called the Integrated Multi-Trophic Aquaculture (IMTA). IMTA has been adopted in many countries such as Canada, Chile, the United States, Japan and China (Troell et al. 1999; Chopin et al. 1999; Buschmann et al. 2009; Zhou et al. 2006). Using seaweeds in this manner very clearly demonstrates their bioremediation potential with respect to nutrients, but their potential for broader bioremediation, and in particular their ability to uptake metals in a polluted estuarine system remains unknown. However, macroalgae species would appear to have many of the features that might make them ideal tools to remove metals from contaminated areas, i.e., relatively short lifecycles, contaminant specificity, and a high capacity for bioaccumulation (Robledo 1993). Given the metal contamination issues in the Derwent, it could be worldwide investigating the use of local

macroalgae species for ‘cleaning-up/ remediation of’ metals from this highly polluted environment.

In order to evaluate the bioremediation capacity of any given seaweed species with respect to metal contamination it is first necessary to understand the metal uptake ability of that species. Previous studies have suggested that the physiological structure of seaweeds might be particularly suitable for metal-biding (Volesky and Holan 1995; Vieria and Volesky 2000), especially those seaweeds with cellulosic components (Vieria and Volesky 2000). The potential for metal uptake has been investigated in a number of seaweed species. Brown (Figueira et al. 2000; Davis et al. 2003) and red seaweed (Gouveia et al. 2013; Felix et al. 2014) are well known for their metal-biding capacity, but less is known about green seaweeds. Evaluating cellular structures (i.e. cell wall) in potential indicators/ bioremediation species could in the first instance, provide evidence of metal biding, metal tolerance, or metal resistance and, as such, provide an assessment of whether a given seaweed species really does show potential to be used for remediation.

The overall goals of this study are to:

1.3 Aims

1) Identify local macrophyte species with potential to be used as biomonitors of metal pollution across a range of habitats found within the Derwent Estuary,
2) Evaluate the potential of these local biomonitors to be used to inform and direct management in metal contaminated areas (within the Derwent estuary and more broadly),

3) Determine whether those macrophytes that are found to be useful as biomonitors could also be used as tools for bioremediation of metal contamination in a polluted estuarine environment, such as the Derwent Estuary,

1.4 Thesis structure

Each research chapter in this study presents original data, and has been written as a manuscript for submission to a refereed scientific journal and adapted to fulfil the University of Tasmania thesis requirements. The organisation of this thesis is presented below:

- **Chapter 1**

  A general introduction provides the context and outlines the main topics covered in this thesis, highlighting the key research questions and goals of the investigation.

- **Chapter 2**

  This chapter examines the broad range of macrophytes throughout the Derwent Estuary, Tasmania, Australia and provides and initial exploration of their potential to be used as bioindicators of heavy metal pollution, with the aim of identifying those species most suitable for biomonitoring.

- **Chapter 3**

  Chapter 3 evaluates the spatial and temporal variability of heavy metals in the macroalga *Ulva australis*, the species identified in Chapter 2 as being most likely to be suitable for biomonitoring, with a view to further determining whether this species could be a candidate for future biomonitoring programs.

- **Chapter 4**

  This chapter describes a new method for examining the ultrastructure of *Ulva* spp. using transmission electron microscopy (TEM).

- **Chapter 5**

  Chapter 5 reports on the growth, photosynthetic (Fv/Fm and ETR_{max}), and ultrastructural responses of *Ulva australis* exposed to three different but ecological relevant levels of zinc (25 $\mu g \cdot L^{-1}$, 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$).

- **Chapter 6**

  This final research chapter reports on a field transplant experiment, where *Ulva australis* was exposed to a gradient of metal pollution at selected sites within the Derwent, in order to evaluate physiological (growth rate), photosynthetic (Fv/Fm and ETR_{max}), and ultrastructural responses under field conditions. The results were used to further determine the applicability of *U. australis* as a biomonitoring and management tool in the Derwent Estuary.

- **Chapter 7**

  This final chapter provides a review of the main findings observed in this research and a general discussion of the broader applications of that information to the area of biomonitoring and bioremediation, highlighting key findings, recommendations, and future research directions.

CHAPTER 2

Macrophytes as bioindicators of heavy metal pollution in estuarine and coastal environments

Preface:

The first chapter outlines for macrophytes species with potential to be used as bioindicators of pollution in a well-known metal polluted environment, the Derwent Estuary. Macrophytes (seaweeds and seagrasses) are popular indicators of pollution, but every environment is unique. Therefore, appropriated local species are required to evaluate pollution in estuarine and coastal ecosystems. Through, a broad-scale survey we identified, from twelve species assessed, three species (*Ruppia megacarpa*, *Zostera muelleri* and *Ulva australis*) with potential to be used as an indicator of metal pollution.

This work was prepared as a manuscript for a refereed journal and adapted for thesis requirements.

2.1 ABSTRACT

The Derwent estuary, in Tasmania (Australia), is highly contaminated with heavy metals with significant levels in both sediments and benthic fauna. However, little is known about metal concentrations in benthic primary producers. We characterised metal concentrations (Arsenic (As), Cadmiun (Cd), Copper (Cu), Lead (Pb), Selenium (Se) and Zinc (Zn)) in twelve species of macrophyte, including red, green and brown algae, and seagrasses, from the Derwent. The metals As, Cu, Pb and Zn were detected in all of the macrophytes assessed, but the levels differed between species. Seagrasses accumulated the highest concentrations of all metals; with Zn levels being particularly high in the seagrass *Ruppia megacarpa* (from the upper Estuary) and Pb was detected in *Zostera muelleri* (from the mid estuary). *Ulva australis* was ubiquitous throughout the middle-lower estuary and accumulated Zn in relatively high concentrations. The findings suggest that analysis of multiple species may be necessary for a comprehensive understanding of estuary-wide metal pollution.

Key words:

Biological indicator, Metal pollution, Seaweed, Seagrasses.

2.2 INTRODUCTION

Biological indicators can provide information on the long-term effects of metal contamination as well as an indication of the potential for impacts at higher levels as a result of trophic interactions. Organisms that accumulate metals in tissues can be particularly informative with species that are consumed by humans (e.g. mussels, fish and oysters) providing important public health understanding of the potential for impacts further up the food chain (Rainbow and Phillips 1993). Using individual organisms to evaluate contaminant loading is known as biomonitoring, and such organism are classed as 'bioindicators' (Zhou et al. 2008). Zhou et al. (2008) proposed five features that would make a species a suitable bioindicator: i) the selected organism should be able to accumulate high levels of pollutants; ii) they should be sessile or constrained to a particular location in order to reflect local pollution; iii) they should be relevant in the food chain; iv) they should be abundant and v) they should be widespread. In addition, practical considerations such as ease of sampling and identification will also help to make a species a good bioindicator (Rainbow and Phillips 1993).

Molluscs, crustaceans and macrophytes have consistently been shown to be valuable cosmopolitan bioindicators (Rainbow 1995; Rainbow and Phillips 1993). Macrophytes, seaweed and seagrasses, have been used in many studies as bioindicators of contamination. A number of studies have used green algae, particularly alga in the Order Ulvales as bioindicators of metal pollution in a number of studies (Ryan et al. 2012; Boubonari et al. 2008; Zbikowski et al. 2007; Brown et al. 1999; Ho 1990). The cosmopolitan distribution and high metal accumulation capacity of *Ulva lactuca*, an extremely common annual green alga, has shown it to be a very valuable bioindicator with a high affinity for manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and lead (Pb),

consequently, this species provides a very good warning of both domestic pollution and industrial contamination (Ho 1990). Several brown algae are also frequently used for biomonitoring. For instance, *Fucus vesiculosus* has been shown to be a popular bioindicator of heavy and trace metals in the north hemisphere (Ryan et al. 2012; Stengel et al. 2005; Guisti 2001; Jayasekera and Rossbach 1996; O'Leary and Breen 1997), *Ascophyllum nodosum* and *Laminaria digitata* have been used to indicate temporal and intraspecific metal fluctuation in a coastal area (Stengel et al., 2005), *Lessonia trabeculata* and *Lessonia nigricens* were effective in identifying Cu mining pollution in Chilean coastal waters (Sáez et al. 2012; Leonardi and Vasquez 1999; Contreras et al. 2009). Red seaweeds have also been shown to be effective in detecting different trace and heavy metals (Ryan et al. 2012; Ródenas de la Rocha et al. 2009; Leal et al. 1997; dos Santos et al. 2014)

Seagrasses are perennial plants, substrate stabilizers and have been shown to be sensitive to pollution, and therefore useful for biomonitoring and as bioindicators of pollution (EPA 2014). They also play a number of important ecological roles; providing a food source, habitat and refuge for marine animals and improving seawater quality by absorbing nutrients through their roots (Papenbrock 2012; Huang et al. 2006). In South Australia, the ecological importance of seagrass communities and the associated risk from coastal pollution has been formally recognised with seagrass being protected in legislation under the Native Vegetation Act (1992).

Some seagrasses are quite specific in their sensitivities to contamination, and their effectiveness as bioindicators of pollution has been clearly demonstrated (Ralph et al. 2006; Tupan et al. 2014; Romero et al. 2006). For instance, *Cymodocea nodosa* has been

used to assess environmental levels of Cd, Mn, Zn, Cu and nickel (Ni) (Llagostera et al. 2011; Nicolaidou and Nott 1998) and *Zostera marina* has been shown to be a useful indicator of temporal variability in concentrations of Cd, Pb, Ni, Mn, Fe, Zn and Cu (Riosmena-Rodríguez et al. 2010). The leaves of *Enhalus acoroides* have been shown to concentrate Cd (Suwandana et al. 2011) whilst *Posidonia oceanica* has been found to accumulate high levels of Zn in its leaves (Schlacher-Hoenlinger and Schlacher 1998). In areas with cobalt (Co), chromium (Cr), Ni, Cd and Pb contamination *P. oceanica* was actually found to be a better gauge of environmental condition than the previously-used popular bioindicator *Mytulis galloprovincialis*, which had previously been used as a key bioindicator (Lafabrie et al. 2007).

The Derwent estuary in southern Tasmania is affected by a broad range of contaminants such as sewage, stormwater, industry, agriculture, and aquaculture inputs (Whitehead et al. 2010) and has been proposed as the one of the most metal polluted systems in Australia (Bloom and Ayling 1977; Whitehead et al. 2010; Wood et al. 1992), possibly even in the world (Jones et al. 2003). Bloom and Ayling (1977) were the first to identify that metallurgical waste from a local refinery was causing the accumulation of metals such as mercury (Hg), arsenic (As), Cd, Cu, Zn and Pb, in the system. The high levels of metals in the sediment are a result of prior industrial practices; zinc and mercury are of particular concern with levels in many parts of the estuary being up to 10 times higher than the Interim Sediment Quality Guidelines (ISQG) ANZECC guidelines levels for (Whitehead et al. 2010). The Derwent Estuary is an important area for recreational fishing and boating activity, as well as marine transport, and therefore the level of contamination presents a threat not only to ecological processes but also to human health.

Amongst the many sources of metal contamination aquaculture is one of the few modern sources (Dean et al. 2007), with fish- feed containing a range of micro-nutrients including Zn (Richardson et al. 1985; Grahn et al. 2001), and antifouling paints used on nets having the potential to increase Cu and Zn loadings in adjacent water and sediments (Macleod and Eriksen 2009). Although it is important to note that, the salmon industry in Tasmania no longer uses metal-based antifouling paints. In Tasmania, salmonid aquaculture is a significant and developing industry, with currently production in the region of 35,000 ton per annum (AUS$ 408.0 millions in 2011) (Skirtun et al. 2012). Several Atlantic salmon farming operations are situated in the D'Entrecasteux Channel, a connected body of water located to the immediate south of the Derwent estuary. In addition, farmed fish can be affected by local water and sediment quality, and so there are also some concerns that the elevated metals levels in the Derwent might adversely affect fish health.

Metal levels in the Derwent sediments, water column, fish and some invertebrates have been monitored for more than 30 years (Bloom and Ayling 1977; Higgins and Mackey 1987; Dix et al. 1975; Whitehead et al. 2010; Ratkowsky et al. 1975) and levels of metals in the water column and surface sediments have been decreasing over recent years, due to improvements in management (Whitehead et al. 2010). However, we still understand very little about the concentration of metals in the biota and in particular in the primary producers within the estuary, or the effects of this on interactive species. Consequently, the aims of this research are:

1. To determine baseline levels in macrophytes of the dominant metal contaminants in macrophytes within the estuary (As, Cd, Cu, Pb, Se and Zn) with samples collected along a spatial gradient of both salinity and contamination

2. To identify macrophyte species with the potential to be used as bioindicators of heavy metal contamination in the Derwent estuary.

2.3 METHODS

- **Study area**

Thirteen study sites were selected in the Derwent estuary, Hobart, Tasmania (42°52’ S: 147°19’ E). The sites were chosen to provide a gradient of both heavy metal impacts and environmental conditions (i.e. poor water quality, industrial discharges, sewage treatment plants, the presence of aquaculture or a recorded history of heavy metals). In addition, less-polluted areas were also sampled as reference sites. Study sites were sampled throughout the Derwent estuary (Fig. 2.1), from Bridgewater in the upper estuary to Sheppards Point at the mouth of North West Bay in the lower estuary, and with samples collected from both sides of the estuary (Table 2.1). The estuary was divided into three regions according to shoreline morphology, salinity, and bathymetry as previously described by (Jordan et al. 2001).

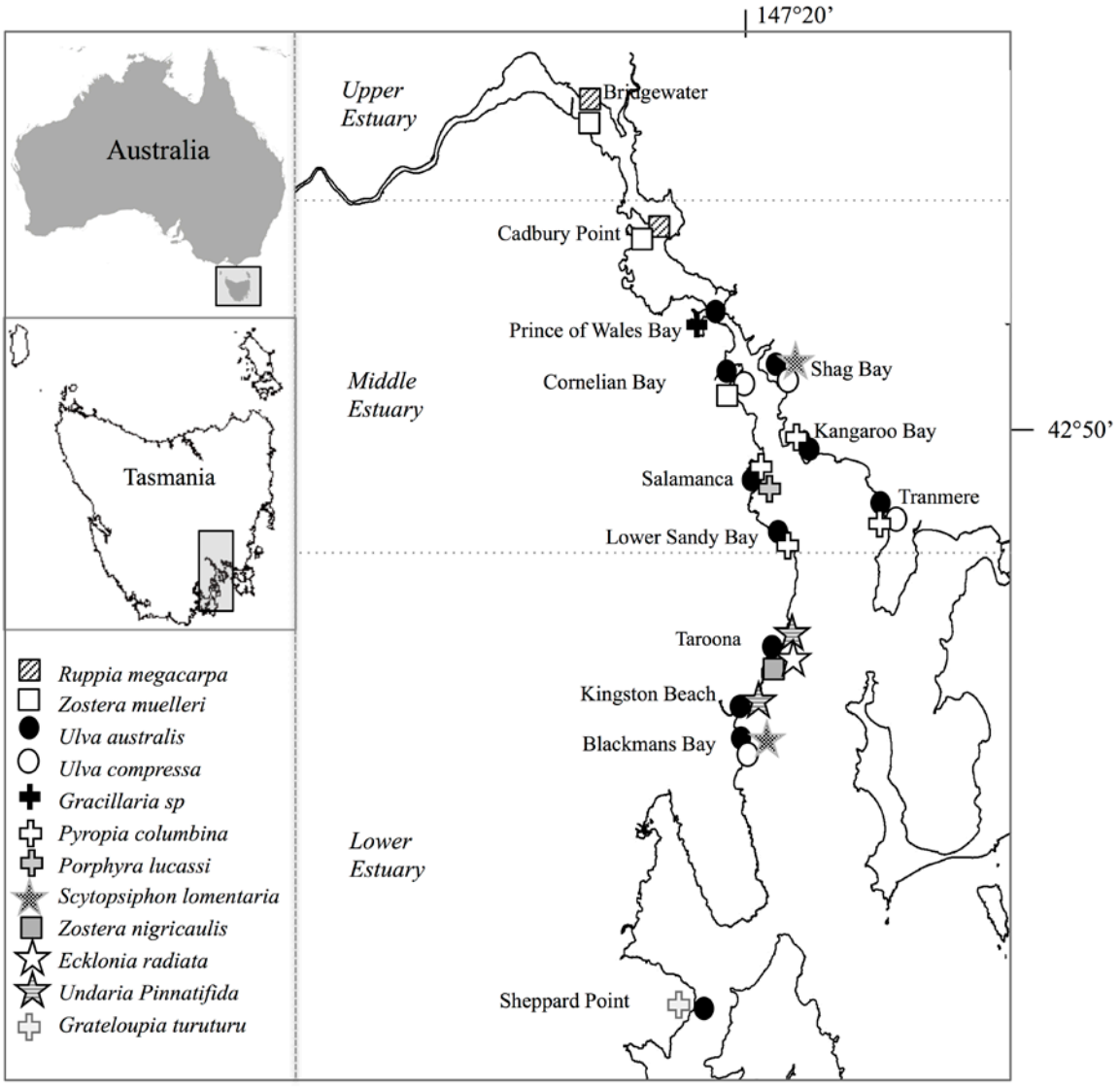

Figure 2.1. Study area showing monitoring sites in Derwent estuary, Tasmania, and the key macrophytes species identified at each site.

Table 2.1. Study sites coordinates thorough the Derwent Estuary.

Code	Site	Latitude (S)	Long (E)
BW	Bridgewater	42°44'26"	147°13'38"
CP	Cadbury Point	42°47'33"	147°15'30"
PWB	Princess of Wales Bay	42°49'50"	147°18'5"
SB	Shag bay	42°50'6"	147°19'54"
CB	Cornelian bay	42°51'10"	147°19'11"
S	Salamanca	42°53'19"	147°20'17"
KB	Kangaroo bay	42°52'29"	147°21'50"
LSB	Lower Sandy bay	42°54'43"	147°18'5"
TRA	Tranmere	42°55'7"	147°24'36"
TAR	Taroona	42°56'29"	147°21'20"
K	Kingston beach	42°58'31"	147°20'9"
BB	Blackmans bay	43°0'0"	147°19'39"
SHP	Sheppard point	43°5'22"S	147°18'15"

- **Macrophyte samples**

Nine species of algae comprising red (*Gracillaria sp., Pyropia columbina* (formerly *Porphyra columbina*), *Porphyra lucassi* and *Grateloupia turuturu),* brown (*Scytopsiphon lomentaria, Ecklonia radiata* and *Undaria pinnatifida)* and green seaweeds (*Ulva australis* and *Ulva compressa)* were sampled. Three species of seagrasses (*Zostera muelleri, Zostera nigricaulis (formerly Heterozostera tasmanica)* and *Ruppia megacarpa* were collected (Fig. 2.1), in each case the whole plant (i.e. homogenised samples of leaves and roots), were analysed.

Sampling was carried out in October 2013. Fresh plant material was collected from the intertidal areas at low tide (n = 3), with the exception of *Z. nigricaulis*, which was collected by snorkelling and samples of *U. australis* and *G. tururu* from the infrastructure around the salmon cages at 2 m depth, which were collected by divers. All samples were transported to the laboratory in resealable plastic bags, and rinsed to remove epiphytes

and sand following the methods of Gledhill et al. (1998). Plants of the same species from each study site were combined to provide a composite sample of approximately 100 g wet weight (WW), seagrasses samples were compounded by leaves and roots. These samples were weighed and dried in an oven at 105 °C for 24 hours to remove all moisture, before being re-weighed to establish a dry weight (DW). Dried material was sent to a certified laboratory, Analytical Services Tasmania (AST), for digestion and metal analysis.

- **Digestion and metal detection**

Dried samples were ground with a mortar and pestle to obtain a particle size of <2 mm. Each 1 g samples was added to a 50 mL digestion tube to which 10 mL concentrated nitric acid (HNO_3 65%) and 10 mL concentrated hydrochloric acid (HCl 30%) were added. Following a 12 hour digestion, the samples were heated to 112.5 °C for 120 min using an Environmental Express™ Hot-block. Upon cooling to room temperature, samples were made up to 50 mL with Milli-Q® water (18 MΩ), filtered using a 10 mL disposable needleless syringe (0.45 µm) before being analysed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) to determine As, Cd, Cu, Pb, Se and Zn levels. Metal detection limits using this approach are 0.1 $mg \cdot kg^{-1}$ wet matter basis (WMB) for As, Cd, Cu, Pb, Zn and 0.5 $mg \cdot kg^{-1}$ WMB for Se. All samples with metals concentration values under the detection limits were considered as the detection limit value. Metal results detected on macrophytes are expressed on dry matter basis (DMB).

- **Concentration Factor**

Bioaccumulation effectiveness was determined by the ratio between concentrations of each metal within the macrophyte thalli and the concentration in seawater, a concentration

factor (CF) (Conti and Cecchetti 2003). This parameter was calculated for 3 species (*Ulva* sp., *Z. muelleri* and *R. megacarpa*), which were selected using the following criteria: i) levels of metal detected within the species (best potential bioindicator of metal pollution) and ii) species with the widest distribution in the Derwent estuary. For this analysis, metals levels were recorded from the surface water (0 m) at each selected site.

$$CF = \frac{Co\ (mg \cdot kg^{-1})}{Csw\ (\mu g \cdot L^{-1})}$$

where: Co is the metal concentration in macrophytes

Csw is the metal concentration in seawater

- **Historical records of water column and sediment metal loading**

The Derwent Estuary Program (DEP, Tasmania, Australia) provided data on metal (As, Cd, Cu, Pb and Zn) levels in surface waters from 2000 to 2014, and levels in surface sediments in 2011. Water column Zn levels are expressed as both the total metal concentration detected in the water column (total metal) and an estimate of the fraction of metal that is bioavailable in the water column (dissolved metal). Marine water quality at the study sites was assessed by the comparison of the DEP data values with the Australian and New Zealand Environment and Conservation Council (ANZECC) trigger values for marine environments (ANZECC 2000). Concentrations of metals in marine waters lower than the 95% trigger levels indicates a value that is expected to protect 95% of species, whereas concentrations lower than the 99% trigger level (the highest protection level, high conservation value) are expected to protect 99% of the species. Different protection levels are applied based on the condition of the ecosystem in question, with the three main categories being ecosystems with high conservation value that are slightly-moderately

disturbed or highly disturbed. Sediment data was assessed against the ANZECC Interim Sediment Quality Guidelines (ISQG) (ANZECC 2000). The ISQG trigger values are based on effects databases for contaminated and uncontaminated sites, and provide a basis for screening sediments based on their contaminant properties. The ISQG-Low level is based on the NOAA effects range-low (10th percentile) value, and the ISQG-High is based on the corresponding median value for each contaminant.

There are no specific guidelines or trigger levels for metal loading in biota per se, although there are guidelines for metal loadings in seafood destined for human consumption. For the purposes of this research, we applied the ANZECC seawater and sediment trigger values as a reference to indicat the potential for chemical stress on the ecosystem.

- **Analysis**

Ulva australis and seagrasses were selected for further data analysis because they were abundant throughout the estuary and contained the highest levels of metals. The correlation between the metal content of these macrophytes and that of both the surface water (values form the DEP for 2013) and the sediment was assessed (Pearson Correlation). Correlation tests were conducted in R studio statistical software (R Core Team 2013). Data are presented as mean ± standard error (SE) of replicates (n = 3).

2.4 RESULTS

- **Metal concentration within macrophytes**

Seagrasses (*R. megacarpa* and *Z. muelleri*) accumulated the highest levels of metals overall (Fig. 2.2), Zn was particularly high (634 ± 12.4 mg·kg^{-1} DMB and 423 ± 1.7 mg·kg^{-1} DMB, respectively). The seaweeds *U. australis* (320 ± 18.3 mg·kg^{-1} DMB), *U. compressa* (177 ± 1.2 mg·kg^{-1} DMB), *Gracillaria sp* (255 ± 8.2 mg·kg^{-1} DMB), *P. columbina* (101.7 ± 0.6 mg·kg^{-1} DMB), *G. turuturu* (126 ± 1.0 mg·kg^{-1} DMB) and *S. lomentaria* (230 ± 3.3 mg·kg^{-1} DMB) each had levels of Zn > 100 mg·kg^{-1} (Fig. 2.2). Arsenic content was greatest in the red alga *P. columbina* (33 ± 0.1 mg·kg^{-1} DMB) from the middle estuary, and the brown seaweed *E. radiata* (54 ± 0.8 mg·kg^{-1} DMB) from the lower estuary. *Zostera muelleri* (111 ± 0.7 mg·kg^{-1} DMB), *Gracillaria* sp. (107 ± 1.7 mg·kg^{-1} DMB) and *U. australis* (76 ± 1.4 mg·kg^{-1} DMB) accumulated the highest levels of Pb, whereas *Ulva compressa.* (33 ± 2.2 mg·kg^{-1} DMB) and *Z. muelleri* (27 ± 0.2 mg·kg^{-1} DMB) had the highest levels of Cu (Fig. 2.2). Zn and Pb were detected at high concentrations in all the species of macrophytes monitored in this research (Zn >600 mg·kg^{-1} and Pb >100 mg·kg^{-1}). Cd and Se levels in were below detection limits in all species studied. Overall metal concentration in macrophytes from highest to lowest order are Zn>Pb>Cu>As.

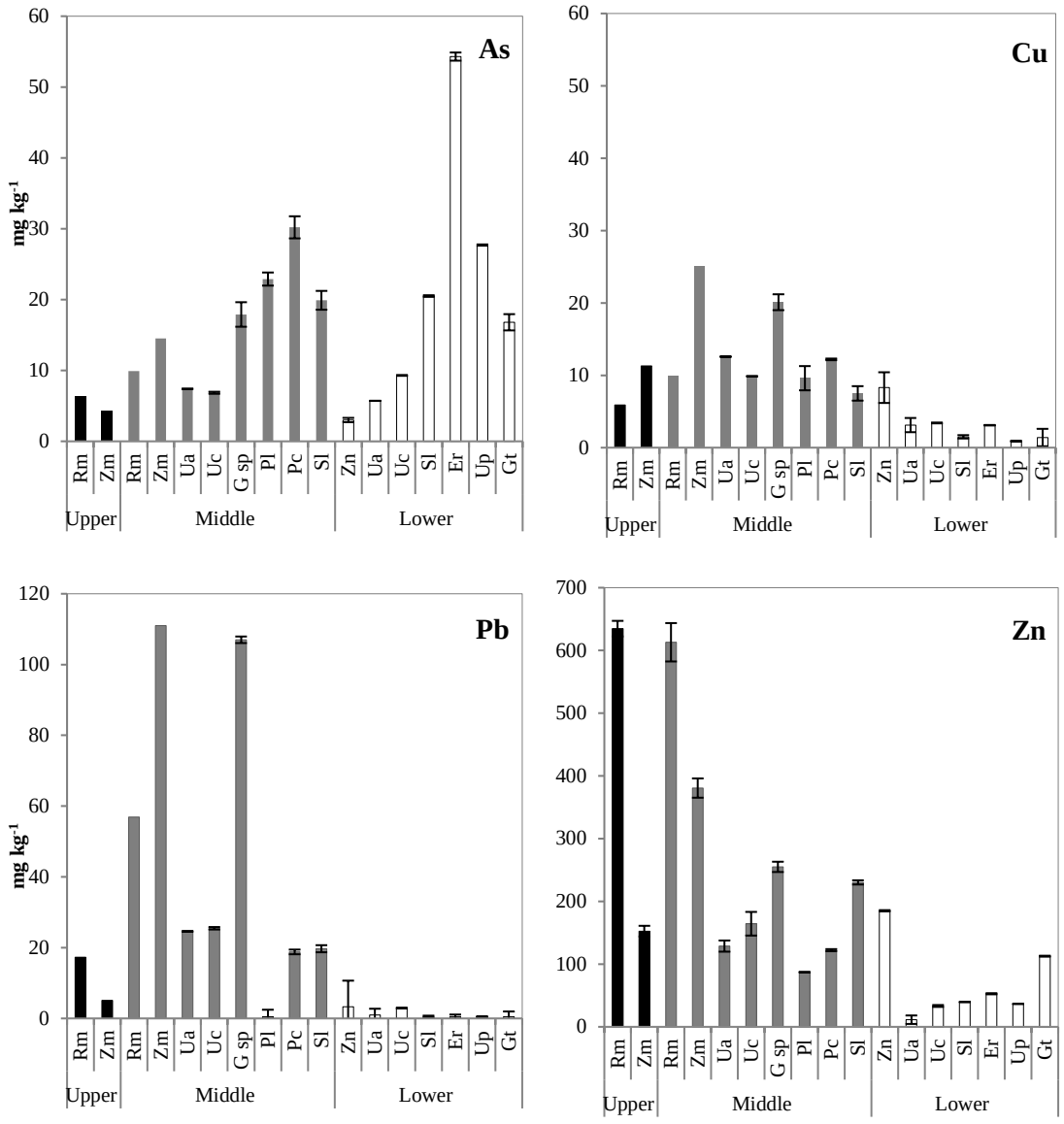

Figure 2.2. Mean metal content (mg·kg^{-1}) of As, Cu, Pb and Zn levels in macrophytes species collected from the Derwent estuary, Tasmania. Rm: *Ruppia megacarpa*, Zm: *Zostera muelleri*, Ua: *Ulva australis*, Uc: *Ulva compressa*, G sp: *Gracilaria* sp., Pl: *Porphyra lucassi*, Pc: *Pyropia columbina*, Sl: *Scytosiphon lomentaria*, Zn*: Zostera nigricaulis*, Er: *Ecklonia radiata*, Up: *Undaria pinnatifida*, Gt: *Grateloupia turuturu*. Note y-axis differs between graphs.

- **Spatial variation in macrophyte metal concentrations**

Metal concentrations varied markedly, both, spatially and between species (Table 2.2). The highest levels of metal accumulation were in macrophyte thalli from the upper and middle estuary i.e. Bridgewater (BW), Cadbury Point (CP), Prince of Wales Bay (PWB)

and Cornelian Bay (CB) (Table 2.2). Three metals in particular (Cu, Pb and Zn), were detected at markedly elevated levels from these sites. Samples collected from Salamanca (S), Tranmere (TRA), Lower Sandy Bay (LSB) and Taroona (TAR) contained the highest concentration of As; these sites are representative of the middle-lower estuary and domestic/urban metals inputs. Samples collected from sites located in the lower estuary i.e. Kingston (K) and Blackmans Bay (BB), contained reduced levels of all metals, indicative of the generally less polluted conditions in this region. Finally, samples collected from Sheppards Point (SHP) in the lower estuary, close to the aquaculture farm had quite mixed results: levels of Zn were low in *U. australis* (10.2 ± 0.2 $mg \cdot kg^{-1}$ DMB) but comparatively high in *G. turuturu* (126 ± 0.7 $mg \cdot kg^{-1}$ DMB) whilst Cu levels were very low in both species (2 ± 0.03 $mg \cdot kg^{-1}$ DMB; 1.4 $mg \cdot kg^{-1}$ DMB, respectively).

Table 2.2. Highest metal content detected on the Derwent estuary and comparison with literature values. Metal content on seaweed and sediment expressed as $mg \cdot kg^{-1}$ Dry Matter Basis (DMB), water metal content expressed as $\mu g \cdot L^{-1}$.

Area/Reference	Site/ Reference	Species/Metal	As	Cu	Pb	Zn
Upper Estuary	BW	*R. megacarpa* [a]	6	6	17	635
		Sediment [b]	36	88	344	980
		Water column [b]	9	2	9	20
Middle Estuary	CP	*R. megacarpa* [a]	10	10	57	613
		Z. muelleri [a]	11	23	68	338
		Sediment [b]	40	77	344	1230
		Water column [b]	-	-	4	21
	PWB	*U. australis* [a]	7	33	77	321
		Gracilaria sp. [a]	18	20	107	255
		Sediment [b]	220	591	1500	9730
		Water column [b]	-	-	-	50
	CB	*U. compressa* [a]	7	11	31	151
		Z. muelleri [a]	18	27	111	424
		Sediment [b]	228	580	1880	14600
		Water column [b]	-	-	3	25
	SB	*U. australis* [a]	7	7	21	168
		S. lomentaria [a]	19	8	20	230
		Sediment [b]	109	257	1090	6440
		Water column [b]	-	25	20	23
ANZECC Trigger values	ISQG High	Sediment	70	270	220	410
	ISQG Low		20	65	50	200
	99%	Water column	-	0.3	2.2	7
	95%		-	1.3	4.4	15
		Aquaculture seawater production	< 30	0.5 - 5	1 - 7	< 5
		Human consumption livestock drinking water*	0.5	-	0.1	20
Literature examples data values	Guisti (2001)	Polluted sediment	-	99	156	112
		Clean sediment	-	12	20	11.3
	Ryan et al. (2012)	*Ulva sp.*	5.7	3.3	1.4	12.2
		P. lanosa	12.3	4.8	3.1	137.6
		Water column	-	1	0.1	1

a: present study results; **b**: data record provided by DEP; *Values expressed on $mg \cdot L^{-1}$

- ***Concentration Factor***

The accumulation efficiency showed that Zn was the metal that was taken up most by macrophytes (seagrasses and *U. australis*) in the Derwent estuary (Table 2.2). However, the concentration factor varied both between species and spatially between sites (Table 2.3). The seagrass, *R. megacarpa* had the highest average CF value overall (CF: 91.98), *Z. muelleri* had a CF of 22.10 and *U. australis* had a CF of 0.60 – 11.75.

Table 2.3. Concentration factor (CF) for macrophytes sampled in this study. Zinc (Zn) content in macrophytes thalli (n = 3), and total Zn content in seawater (n = 4) by study site, together with published CF values for selected species.

Study site	Macrophyte species	Macrophytes Zn (mg·kg^{-1})	Sea water Zn (µg·L^{-1})	CF
BW	*Ruppia megacarpa* [a]	634.7	6.90	91.98
	Zostera muelleri [a]	152.5	6.90	22.10
CP	*Ruppia megacarpa* [a]	613	12.10	5.66
	Zostera muelleri [a]	337.5	12.10	27.89
PWB	*Ulva australis* [a]	320.7	27.30	11.75
SB	*Ulva australis* [a]	177.7	18.40	9.66
KB	*Ulva australis* [a]	97	14.50	6.69
TAR	*Ulva australis* [a]	7.1	11.70	0.60
Literature References	*Cymodocea serrulata* [c] *(leaf)*	0.05 -0.54	0.03-0.060	1.00 – 11.0
	Syringodium isoetifolium	0.15 – 0.59		0.440 –11.0
	Ulva australis [d]	12.2	1	12 200
	Ulva I [e]	45	9.3 ± 1.58	5 010

a: present study; **c**: Govindasamy et al., 2011; **d**: Ryan et al., 2012; **e**: Conti and Cecchetti, 2003

- **Historical records of water column and sediment metal loading**

The long term (9 years) monitoring data from the Derwent estuary shows that the surface waters from the middle estuary (i.e. Cadbury Point, Prince of Wales Bay and Shag Bay) had the highest levels of Cu, Pb and Zn (Table 2.2). Prince of Wales Bay had the highest concentrations, Zn <50 µg·L^{-1} in surface seawater. Sites in the lower estuary (i.e. Kingston Beach and Blackmans Bay) generally had lower levels of all metals (total Zn <12 µg·L^{-1} for both locations). Dissolved Zn levels were at consistent with Total Zn observed throughout the estuary (Fig. 2.3). Arsenic was detected in the Derwent's surface waters from 2000 until 2005, but this metal has not been monitored since 2005.

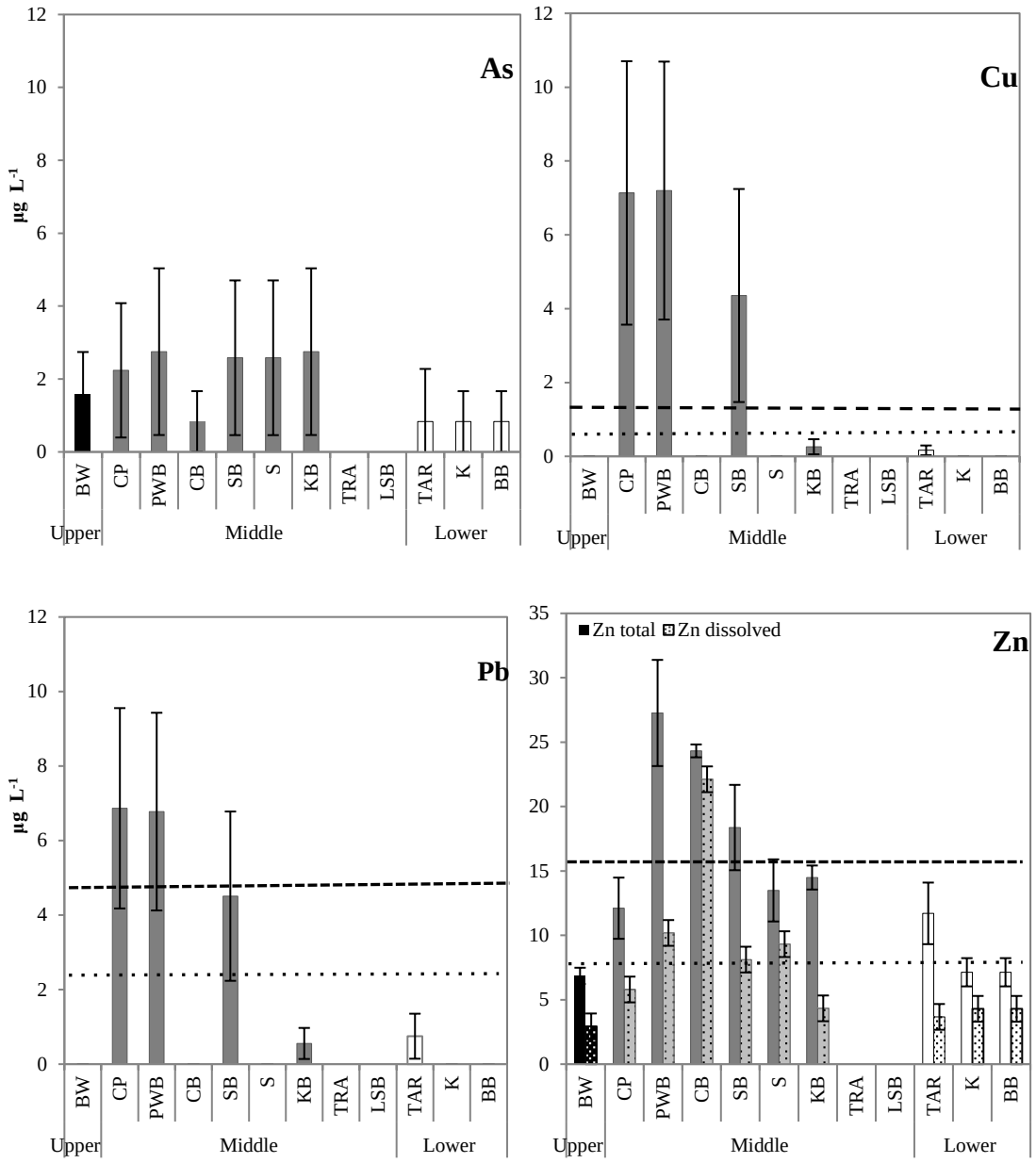

Figure 2.3. Content (µg·L^{-1}) of arsenic (As), copper (Cu), lead (Pb) and zinc (Zn) in the surface waters for each of the study sites throughout the Derwent estuary. Values are expressed as means of total metal content ± SE. Content of As, Cu and Pb are based on the average of all reported measurements from 2005 to 2014, and As is based on the average of levels recorded between 2000-2005. Zinc content are shown for both total and dissolved Zn. Site abbreviations indicate BW: Bridgewater, CP: Cadbury point, PWB: Princess of Wales Bay, CB: Cornelian Bay, SB: Shag Bay, S: Salamanca, KB: Kangaroo Bay, TRA: Tranmere, LSB: Lower Sandy Bay, TAR: Taroona, K: Kingston Beach, BB: Blackman Bay. Horizontal dashed-lines represent ANZECC trigger values for protection of 95% and horizontal dotted-lines 99% of species, note that there is no trigger value for arsenic in marine water. Note y-axis differs between graphs. (Data courtesy of Derwent Estuary Program)

Zn contamination of the sediments is widespread throughout the estuary, but levels are particularly high in the middle estuary (Cornelian Bay) with the concentrations in excess of 14,000 $mg \cdot kg^{-1}$ (Whitehead S, pers.comm.). Lead was also markedly elevated in the mid-estuary (Cornelian Bay) with a maximum concentration of 1,880 $mg \cdot kg^{-1}$. The middle estuary (Prince of Wales Bay, Cornelian Bay and Shag Bay) was consistently the most metal contaminated region, with sites in the lower estuary (Kingston Beach and Blackmans Bay) being markedly less polluted (Fig. 2.4). Over the period that the Derwent Estuary has been monitored (2000 to 2015) Zn and Pb levels in sediments have decreased, although there are still over ISQG guidelines limits there has clearly been some improvements (Coughanowr et al. 2015). Copper level in the sediments around the salmon farm (Sheppard Point), were consistently below the ISQG-high trigger level (ANZECC 2000), and have been declining since 2008 (i.e. from 71.5 ± 23.8 $mg \cdot kg^{-1}$ in 2008 to 28.5 ± 32.8 $mg \cdot kg^{-1}$ in 2010, (Barrenger M, pers.comm.)), potentially in response to the removal of anti-fouled nets.

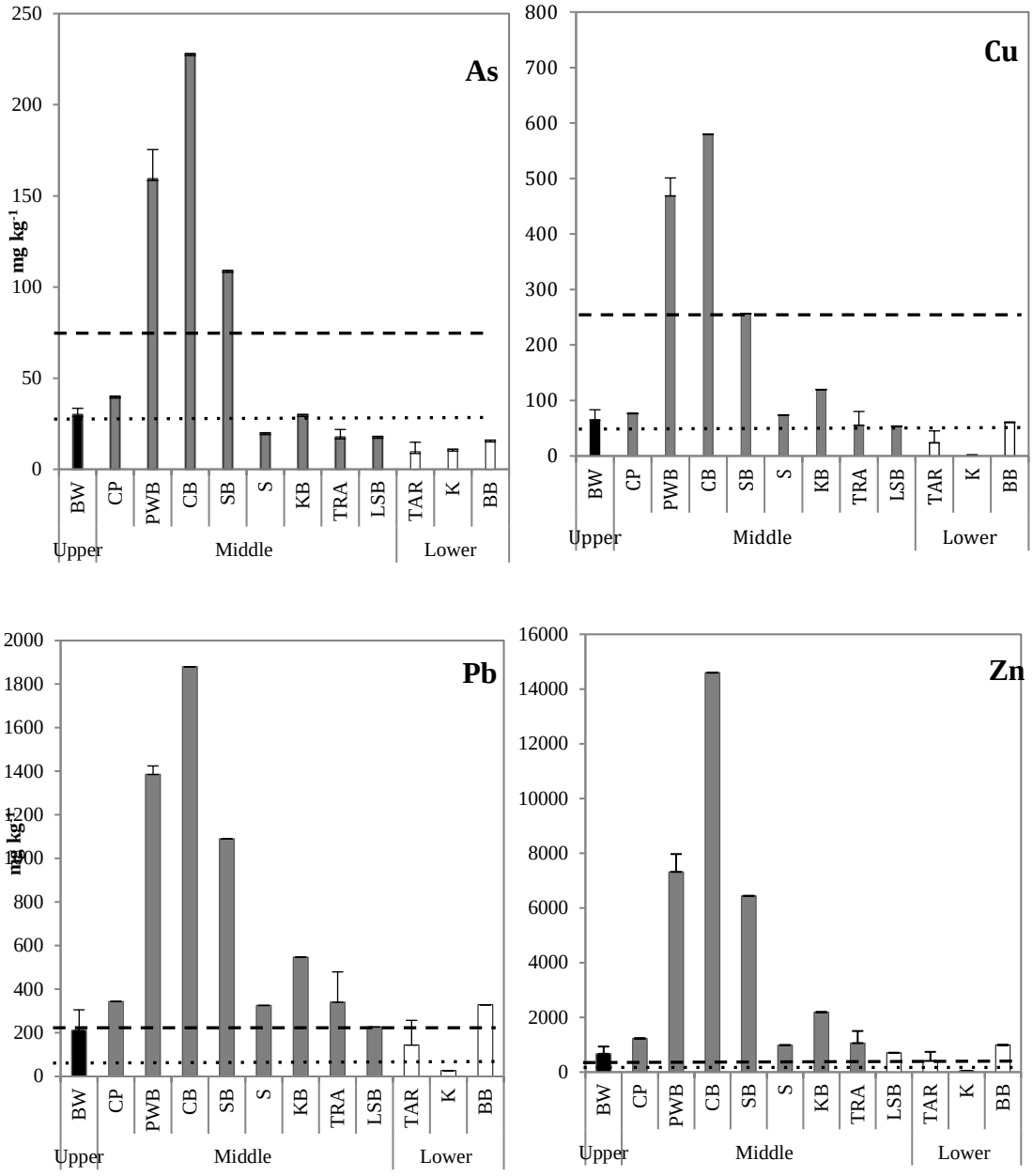

Figure 2.4**.** Sediment metal content (mg·kg^{-1} of Dry Matter Biomass, DMB) for the Derwent estuary sampled sites in 2011. Metal expressed as means ± SE, (*n* = 3). Sites codes BW: Bridgewater, CP: Cadbury point, PWB: Prince of Wales Bay, CB: Cornelian Bay, SB: Shag Bay, S: Salamanca, KB: Kangaroo Bay, TRA: Tranmere, LSB: Lower Sandy Bay, TAR: Taroona, K: Kingston Beach, BB: Blackman Bay. Horizontal dashed-lines indicate the ANZECC ISQG High and dotted-lines indicate ISQG Low trigger levels for each metal respectively. Note y-axis differs between graphs. (Data courtesy of Derwent Estuary Program).

For *U. australis*, there was a significant positive correlation between levels of As, Cu, Pb and Zn in combination and the background environmental loading for both seawater ($r = 0.88$, $p < 0.05$) and sediments ($r = 0.95$, $p < 0.05$). However, the relationship between sediment and water column loading and *Ulva* sp. content was less clear when each metal was considered independently. Metal levels in seagrasses were significantly correlated with sediment metal concentration ($r = 0.82$, $p < 0.05$), and in this case, a similar pattern was also observed when the correlation was conducted for the individual metals (As, Cu, Pb and Zn). However, there was no correlation between seagrasses metal content and seawater ($r = 0.77$, $p < 0.05$).

2.5 DISCUSSION

A clear gradient of metal concentration was detected in the macrophytes sampled, with the highest concentration of the four main metals (As, Cu, Pb and Zn) decreasing from the upper to lower estuary. Concentration of metals varied in macrophytes species sampled across the estuary, with three of the twelve species studied showing potential to be used as bioindicators of metal pollution in the Derwent. Identifying potential macrophytes species to detect heavy metal pollution is a useful approach for supporting biomonitoring programs in a large ecosystem with strong gradients of metal concentration.

Zinc was the most prevalent metal in the Derwent, and principally enters the environment as a result of the smelting and refining of Zn in the middle estuary (Jones et al. 2003). The levels of Zn observed in one species of seagrass (*R. megacarpa*) were the highest recorded for this group of macrophytes from the Derwent. Although, Zn is an essential

micronutrient for some organisms, at high concentrations it can reduce growth, compromise photosynthesis and even kill algae; Zn has been shown to be toxic to seven species of algae even at concentrations as low as 6.538 $\mu g \cdot L^{-1}$ (Baumann et al. 2009). Concentrations of Zn detected in surface waters of the Derwent were up to 25 $\mu g \cdot L^{-1}$. Close to the Salmon farm, the levels of Zn in macrophytes were relatively high, suggesting that there is some release either from the cage infrastructure or via feed input.

Z. muelleri and *Gracillaria* sp. concentrated high levels of Pb, which can be toxic and produce adverse effect in some organisms. However, the highest concentrations in these two species from the middle estuary were <110 $mg \cdot kg^{-1}$, which is considerably less than has been previously described for similar macrophytes (*Padina pavonica* and *Cladophora albida)* from the northern hemisphere, where levels approaching 500 $mg \cdot kg^{-1}$ have been reported (Malea et al. 1995). Adverse effects of Pb have been observed at 10 $mg \cdot L^{-1}$, with significant impacts on algal growth in Fucales, whilst Pb has been shown to be toxic at 200 $mg \cdot L^{-1}$ (Hurd et al. 2014). Nevertheless, bioaccumulation of Pb is a real concern and particularly the potential to transfer Pb to the food chain, and the implications for human health (Baumann et al. 2009; Souza et al. 2012).

The two main metals, Zn and Pb, were found in high concentration in the middle estuary, with levels particularly elevate, closer to the Zn smelter and where the sediment was particularly fine/silty (<63 µm). Mucha et al. (2004) suggests that sediment grain size is a strong determinant of heavy metal accumulation, with fine sediments tending to bioaccumulate metals, especially the finest fraction (mud size) <63 µm (Guisti and Zhang 2002; Guisti 2001; Jones et al. 2003). The highest metal concentrations in macrophytes were from those sites previously described by Whitehead et al. (2010) and Jones et al.

(2003) as the most polluted areas i.e. Cadbury Point, Cornelian Bay, Prince of Wales Bay and Shag Bay, middle estuary. Metal levels in macrophytes from the lower estuary were consistent with those described elsewhere as being from an unpolluted environment. Ryan et al. (2012) reported Zn <13 $mg \cdot kg^{-1}$ DMB and other metals such as As, Pb and Cu < 5 $mg \cdot kg^{-1}$ DMB in *Ulva* sp. sampled from unpolluted environment. While, Zn <17 $mg \cdot kg^{-1}$ was detected in the brown seaweed *Fucus vesiculosus* that was collected from a clean environment, concentrations >1015 $mg \cdot kg^{-1}$ were found in polluted areas impacted by urban and industrial activities Guisti (2001). On this basis, the lower estuary could be categorised as an unstressed zone of the Derwent, together with the aquatic flora from this area.

Many of the seaweeds species assessed in this study had the ability to accumulate heavy metals. *U. compressa, Gracillaria* sp and *S. lomentaria* concentrated high levels of Zn, and *Gracillaria* sp. accumulated high levels of Pb, but these species were restricted distributed. Species with limited distribution could be useful bioindicators for monitoring those specific locations. While (Edmonds and Francesconi 1981) noted that *E. radiata* was the major As accumulator in western Australian coastal ecosystem, in the Derwent Estuary *E. radiata* accumulated As at 54 ± 0.8 $mg \cdot kg^{-1}$, and its distribution was restricted only to the lower estuary where As concentrations are very low. As a result, in the Derwent *E. radiata* cannot be considered as a good bioindicator of metal pollution of the estuary system. This result seemed inconsistent with the sediment data, which suggested that As concentrations were low in this area, <20 $mg \cdot kg^{-1}$ (Whitehead et al. 2010), and As has not been detected in the water column throughout the estuary since 2005. Cu was also only detected at very low levels in macrophytes throughout the estuary even around the

salmon farm (<2.0 $mg \cdot kg^{-1}$), which suggests that there is no contamination by Cu in this area.

Three macrophytes species stand out as having the potential to be useful indicators of heavy metal pollution in the Derwent estuary. *U. australis*, *Z. nigricaulis* and *R. megacarpa*. Each of them have different advantages. *Ulva australis* meets many of the criteria described earlier; it accumulates high levels of pollutants, is sessile, relevant to the food chain, abundant, easily collected and has a widespread distribution within the area of interest. It is therefore a very good candidate species for biomonitoring in this system. However, it also has a major disadvantage in that is an annual species, and thus can be only ever used to provide seasonal information about each location studied. The seagrasses, *R. megacarpa* and *Z. muelleri*, also accumulated high levels of metals, in fact for many metals, particularly Zn, they were the highest measured in this investigation. Although, restricted to the upper reaches of the estuary, these seagrasses can provide information on the longer-term accumulation of metals in their tissues and might better represent the health status of the environment.

To verify the metal uptake ability of my potential bioindicators we investigated the relationship between tissue concentrations and concentration in the surrounding environment (water and sediment). There was a strong correlation between the metal levels in *U. australis* and the environmental load of Zn, both in the water column and in the sediments. The relationship between tissue metals content and water colun concentration of that metal might be expected given that seaweeds take up nutrients from the water column, and therefore would similarly concentrate metals ions from seawater (Hurd et al. 2014; Lobban and Harrison 1994). The strong correlation between the metal content of *U. australis* and sediments is explained by the exceptionally high metal

concentrations in the sediments, which were much higher than in the water. *Ulva* grows in intertidal areas where the water motion is more likely to resuspend sediment and increase the metal bioavailability. There was a weaker correlation between the Zn content in the two seagrass species, *R. megacarpa* and *Z. muelleri,* and the metal levels detected in seawater, regardless of whether the whole plant, the roots and the leaves, were sampled. A stronger correlation was found between seagrasses and sediments. Previous studies have suggested that both nutrient and metal uptake in seagrasses occurs in the leaves and vascular tissue, and as a result, any major metal affinity would be with leaves rather than roots (Ambo-Rappe et al. 2007; Malea and Haritonidis 1989; Romero et al. 2006). Fabris et al. (1982) demonstrated that *H. tasmanica* leaves uptake up to 1.8 $mg \cdot kg^{-1}$ of Cd and roots-rhizome 0.4 $mg \cdot kg^{-1}$, although it is important to note that the results were obtained from the plants grown in non-contaminated sediments. Carter and Eriksen (1992) also found that leaves of *Z. muelleri* uptake greater amounts of Cu than roots-rhizome in a laboratory experiment, and similarly Ward (1987) demonstrated that leaves of the seagrass *P. australis* collected during spring close to a lead smelter are an effective bioindicator of Zn (4, 241 $mg \cdot kg^{-1}$ DW), Cd (541 $mg \cdot kg^{-1}$ DW) and Pb (354 $mg \cdot kg^{-1}$ DW). The results of the current study suggest that further investigation on *R. megacarpa* and *Z. muelleri* is warranted to better understand the physiological responses and uptake variability of both the roots and leaves. According to the results obtained in this investigation and the historical metal records for the estuary, it would seem that seagrasses from the Derwent may be pre-adapted to elevated levels of metal, and are able to tolerate and resist the high concentrations of Zn that exists in the environment, making these seagrasses good bioindicators of metal pollution.

The effectiveness of these species as bioindicators could be corroborated by examining

the bioaccumulation ability as indicated by the concentration factor CF (Conti and Cecchetti 2003). CF values for *U. australis* in the current study ranged from 0.604 to 11.746 depending on location within the estuary. This is consistent with the range of values previously described for Ulvales in the literature (Table 2.2) (Ryan et al. 2012; Conti and Cecchetti 2003). This confirms that *U. australis* would be a good bioindicator of metal pollution in coastal and estuarine waters, as it has been demonstrated in other Ulvacean (Conti and Cecchetti 2003; Jayasekera and Rossbach 1996; O'Leary and Breen 1997; Malea and Haritonidis 2000; Ho 1990; Favero et al. 1996). CF values for the seagrasses, *R. megacarpa* and *Z. muelleri,* were comparable to levels observed in other macrophytes species such as *Ascophyllum nodosum* (CF: 80 900) and *Polysiphonia lanosa* (CF: 137 600) (Ryan et al. 2012), and were much higher than other seagrasses species (*Cymodocea serrulata:* 1 – 11) and *Syrngodium isoetifolium*: 0.4 – 11) (Govindasamy et al. 2011). This suggests that *R. megacarpa* and *Z. muelleri* would also be good bioindicators of metal pollution on estuarine waters.

Although the results obtained in this research shown that macrophytes from the Derwent are strongly impacted by metal pollution, it seems that in general the flora is tolerant to these conditions. However, further investigation is needed to evaluate major metal effects on associated organism, and transfer to the food chain. For instance, Roberts et al. (2006) demonstrated that the number of associated epifauna is reduced in brown seaweeds contaminated by Cu. It is also possible that high levels of heavy metals contamination, of the scale detected in this investigation, could affect the normal reproduction, physiology or growth of Derwent's primary producers. The literature reports that early stages of *Macrocystis pyrifera* are sensitive to metals such as Zn (Anderson and Hunt 1988). While,

Chung and Brinkhuis (1978) discovered that Cu affected, in different grades, early stages of the brown seaweed *Laminaria saccharina*.

2.6 CONCLUSIONS

This study provides a descriptive spatial distribution of four main metals (As, Cu, Pb and Zn) detectable in macrophytes species. Metal content decreased from the upper to lower estuary with the highest levels recorded in a number of macrophytes species located in the historically most contaminated areas of the Derwent estuary.

Seagrass showed the highest level of metals detected in this study contributing to long-term history of pollution. However, an annual species such as *U. australis* is a valuable tool for broad monitoring system as the Derwent estuary.

We conclude that three of the twelve macrophytes species assessed, *U. australis, Z. muelleri* and *R. megacarpa* have the potential as bioindicators of heavy metal pollution, to be considered for future biomonitoring programs in the Derwent estuary.

CHAPTER 3

Ulva australis as a tool for monitoring metal-polluted estuarine system; spatial and temporal considerations

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Preface:

This research chapter covers spatial and temporal considerations for assessing *Ulva australis* as a biomonitoring tool of metal pollution. Throughout three years of monitoring. I observed that there was a strong spatial distribution of the metal content in *U. australis*. *U. australis* demonstrated great potential for inclusion in biomonitoring programs for improve managements.

This work has written as a manuscript for a refereed journal and modified for thesis requirements, and submitted as a conference paper in the 15th International Conference on Environmental Science and Technology Rhodes, Greece

3.1 ABSTRACT

This study investigated temporal and spatial patterns of heavy metal content in *Ulva australis*. Samples were collected from the Derwent Estuary, Tasmania, Australia, over 3 years (2013-2015) at locations where historically arsenic, cadmium, lead and zinc were high in sediments and seawater. Zinc and lead content were high in *U. australis* at all sampling times, with levels consistent with the spatial distribution of metals within the estuary. Zinc in *Ulva* varied seasonally (4.8 – 320.7 $mg \cdot kg^{-1}$), but lead did not. Zinc and lead were highest in the middle-upper estuary, close to the zinc smelter, where seawater concentrations were higher. The results suggest that spatial variation of metal content in *Ulva* is a reflection of variability in the seawater, which in turn indicates that *U. australis* could be used for monitoring the effects of metals in estuarine systems, and that *U. australis* could be a useful addition to existing management strategies.

Key words:

Biological indicators, Contamination, Macroalgae, Seasonal variability, *Ulva*.

3.2 INTRODUCTION

Metal pollution in aquatic environments is a worldwide concern. Contamination of estuaries and coastal zones is increasing, and consequently, this may have detrimental effects on the flora and fauna in those systems (Lionetto et al. 2012; Kennish 2002). Biological monitoring or biomonitoring is one way to obtain information on the potential effects of metals pollutants (Zhou et al. 2008), and a better understanding of the ecological condition of the environment (Campbell 2002). However, for biomonitoring to be effective, reliable indicators of pollution are needed (Conti et al. 2002); a suitable bioindicator can provide important information on the ecological effect of heavy metal loading in the surrounding environment (Rainbow 1995; Rainbow and Phillips 1993; Zhou et al. 2008; Conti et al. 2002).

Urbanised estuaries are often affected by a wide range of anthropogenic perturbations including metal contamination (Kennish 2002), and this can result in major ecological changes in the system (O'Leary and Breen 1997; Bryan et al. 1985; Ho 1984; Coughanowr et al. 2015; Bryan and Langston 1992). Monitoring of such estuaries often relies on a combination of both chemical (seawater and sediment) and biological measures. Whilst, chemical measures of contaminants levels in seawater can be very precise (Campbell 2002), they can only offer a snapshot of the conditions at the time of sampling. Sediments, while more temporally stable, are highly heterogeneous and as such can vary widely with depth or sediment type. Consequently, biota may offer a better predictor of overall ecosystem health, as they will provide both a specific understanding of biological uptake and its impacts together with an integrated indication of environmental conditions over time (Holt and Miller 2011).

Understanding the temporal and spatial variability of the total metals loads within a system is important, but it is also important to understand the bioavailability (the proportion that is available for uptake and accumulation) and as such biomonitoring programs are essential (Rainbow 1995). Spatial and temporal assessment can provide accurate information on episodic discharges (i.e. industrial discharge) and how these might affect the system, as well as information on the effect of changes in natural and biological processes (i.e. estuarine-scale changes). Understanding how metal loads vary naturally in a chosen bioindicator is essential baseline information if we are to distinguish the effects of anthropogenic inputs (Rainbow 1993), and is in fact a specified pre-requisite for recommending a bioindicator species for biomonitoring programs.

This is a developing field of research but there are some cosmopolitan bioindicators of metal pollution (i.e. invertebrates and seaweeds), which have already been shown to accumulate metals and provide time-integrated measurements of pollution in aquatic systems (Rainbow and Phillips 1993). Seaweeds are often used, and have been recommended by a number of authors as valuable bioindicators of metal pollution (Rainbow 1995; Rainbow and Phillips 1993). They have a number of attributes which make them particularly suitable as bioindicators. In particular, many species show a high tolerance to metal pollution and, being static, they will reflect the conditions at a given location and are easy to sample (Zhou et al. 2008).

However, the ability of seaweeds to take up metals can be influenced by biological factors, such as growth rate and seasonal die backs (Malea and Haritonidis 1999b), but also take into account broader seasonal changes in environmental conditions that might confound metal uptake. Seasonal patterns are dependent not only on the seaweed species,

but can also be affected by the particular metal of interest and its concentration, the metal input source and any episodic considerations for that input and, of course the season (Malea et al. 1995; Brown et al. 1999; Riget et al. 1995). Consequently, when employing seaweeds as a tool for biomonitoring, it is important to understand how all of these factors might influence the response (i.e. metal uptake rate and total load) of any chosen species (Riget et al. 1995). Green seaweeds, such as members of the order Ulvales, are often used in metal biomonitoring investigations (Brown et al. 1999), mainly because they are widespread in many systems and have also been shown to take up metals in high concentrations. *Ulva lactuca* and *U. rigida* are very well established as bioindicators of metal pollution (Ho 1990; Favero et al. 1996; Malea and Haritonidis 2000).

The Derwent Estuary in Tasmania, Australia, is a highly metal-polluted system (Bloom and Ayling 1977) with zinc (Zn) being the most significant contaminant, particularly in the middle estuary (see in Chapter 2, figure 2.2, 2.3 and 2.4), due to inputs from a refinery (Coughanowr et al. 2015). The Derwent Estuary Program (DEP) has actively monitored the estuary for the past 15 years; with water column metals being assessed at a number of fixed points throughout the estuary regularly throughout the year. Two detailed surveys of sediment metal concentrations having been undertaken (Coughanowr et al. 2015; Whitehead et al. 2010) together with a number of independent surveys assessing metal loads in biota. The DEP monitoring program and other research has also shown that arsenic (As), cadmium (Cd) copper (Cu), lead (Pb) and Zn are all present at elevated concentrations in sediments (Coughanowr et al. 2015; Bloom and Ayling 1977; Wood et al. 1992), shellfish (Bloom and Ayling 1977; Eustace 1974) and fish (Verdouw et al. 2010; Dix et al. 1975; Ratkowsky et al. 1975; Langolis et al. 1987; Eustace 1974).

However, there is no information on the content of heavy metals in seaweeds in the Derwent.

In Chapter 2 it was established that *Ulva australis* is widely distributed throughout the Derwent estuary, and that there are spatial differences in metal levels that appear to be correlated with environmental loadings, which would suggest that *U. australis* could potentially be useful as a biomonitor. However, in order to establish this, it is important to understand how temporal changes, both inter-annual (longer-term) and seasonal (short-term) might affect metal accumulation.

Subsequently, this investigation aims to:

1) Assess the spatial and temporal variation of metal content in *Ulva australis* within the Derwent Estuary over the period between 2013 to 2015.
2) Determine the effectiveness of *Ulva australis* as a tool for biomonitoring.

3.3 METHODS

- **Study area**

Eight study sites were selected from the Derwent Estuary (Fig. 3.1). Sites were selected to reflect different heavy metal loads and sources of pollution, i.e. levels of industrial discharge, sewage treatment levels, and heavy metal concentrations observed in chapter 2. The estuary was divided into four regions, according to spatial analysis undertaken in the present study and based on division according to 015(Coughanowr et al. 2015). Study sites were located on both sides of the Estuary, and regions were defined as follows: middle-upper estuary (PWB and SB, n = 2), middle-lower estuary (KB, TRA, LSB, n = 3), and lower estuary (TAR, K, BB, n = 3).

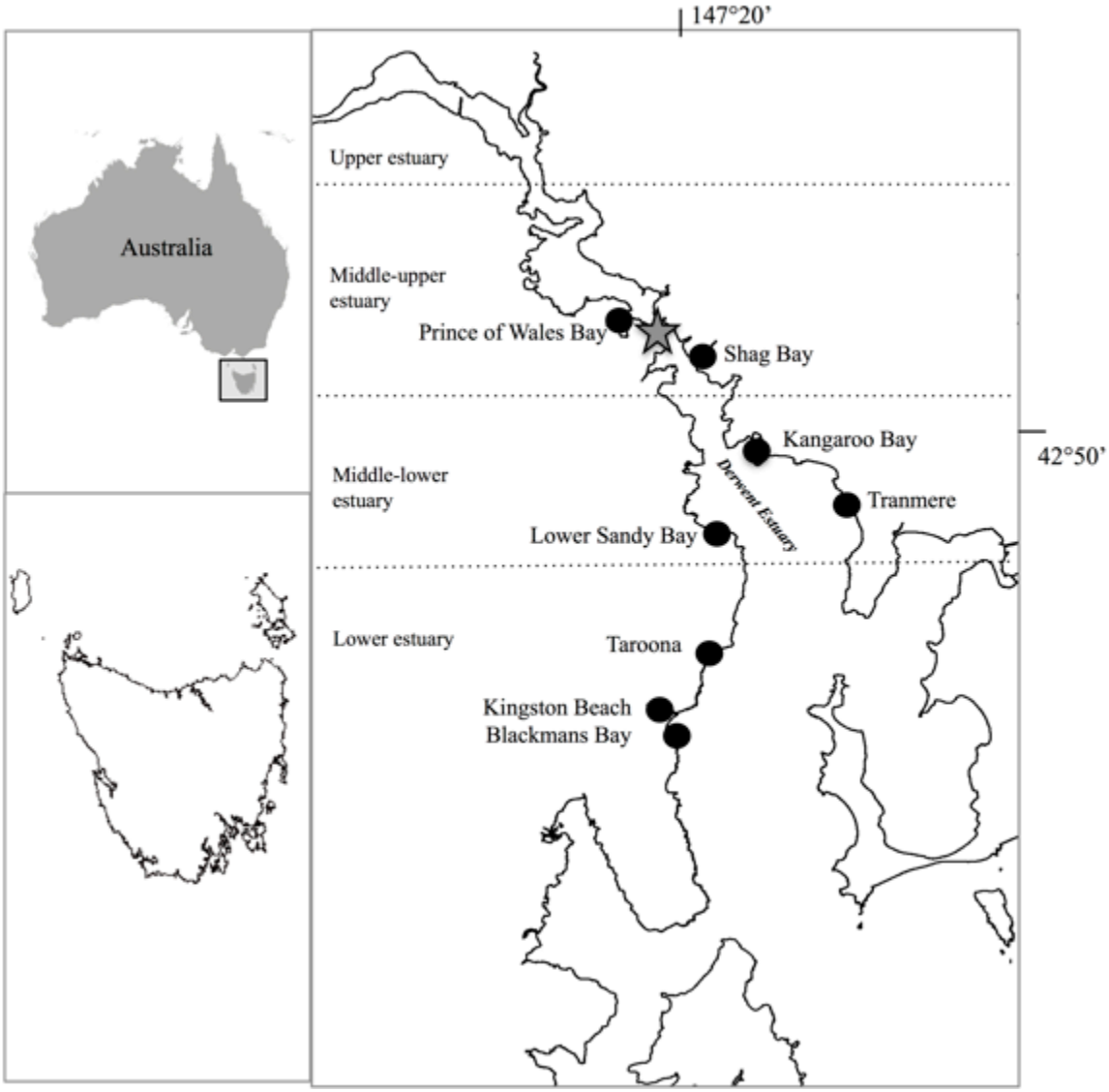


Figure 3.1. Study area and sites in the Derwent Estuary, Tasmania, Australia based on results of the spatial analysis undertaken in the present study. Grey star indicate zinc smelter location on the estuary.

- **Field collections**

To evaluate inter-annual variability, samples of *U. australis* (n = 3) were collected on the 13th and 15th of October 2013 (spring 2013) and again on the 13th and 14th of October 2015 (spring 2015) from each of the eight study stations in the Derwent Estuary. For seasonal variability assessment, *U. australis* thalli were collected from all study sites every three months from October 2013 to October 2015.

Samples were collected from intertidal areas at low tide. Approximately 50 g of *U. australis* was placed in sealable plastic bags with 3 replicate samples collected at each study site. Samples were stored and transported to the laboratory in an insulated container. In the laboratory, samples were rinsed with Milli-Q® water (18 MΩ) and 10% ethanol to remove any epiphytes, salt and sand prior to being weighed to obtain the initial wet weight (Wi) and then frozen at -24 °C until required for further analysis.

- **Metal analysis**

Frozen samples were freeze-dried (FreeZone 4.5 Labconco) for 48 h until constant weight and a final dry weight was recorded (Wf). The sample was then ground using a coffee grinder to obtain a fine and relatively homogenous sample (<2 mm particle size) for metal analysis. Samples were analysed by Analytical Services Tasmania (AST) laboratory, a NATA (National Association of Testing Authorities, Australia) certified laboratory, for detection of Pb and Zn using an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Metal detection limits for Pb and Zn were 0.1 $mg \cdot kg^{-1}$ Dry Matter Basis (DMB).

The Derwent Estuary Program (DEP) provided total zinc concentration ($\mu g \cdot L^{-1}$) in surface waters for the study sites located at the middle estuary (PWB: Princes of Wales Bay, SB: Shag Bay, KB: Kangaroo Bay, TRA: Tranmere, LSB: Lower Sandy Bay). Total Zn for the lower estuary was under detection limits; Zn 1 $\mu g \cdot L^{-1}$. Sampling was conducted once per month at 0.1 m depth and samples analysed for total zinc by Analytical Services Tasmania (AST). It is important note that the DEP water column monitoring, since 2009 only measures total Zn in waters, but that analysis of earlier data showed a strong correlation between the dissolved and the colloidal and particular phases, with 85% of the

total Zn measured being in the dissolved form. Whilst the measure is an aggregate, the proportional relationship was consistent between sites and as such, it is appropriate to compare with the metal loads observed in *U. australis*.

- **Statistical analysis**

To determine spatial differences in metal content in *U. australis*, a two-way ANOVA (95% confidence interval) with site and metal as factors was used to evaluate differences in seaweed metal content at each of the study sites in spring 2013. A Tukey's HSD test was performed post-hoc to further differentiate site interactions and metal interactions. Analysis was carried out with SPSS IBM Statistic version 22.

To assess inter-annual differences in metal content in *U. australis* between spring 2013 and spring 2015, a general linear mixed model (GLMM) with area as random effect and year as factor, and gamma distribution, was applied to the data. Where this showed a significant interaction between site and time, pairwise contrasts were undertaken using a Least-Square Means (lsmeans) package (Lenth and Hervé 2015), with Bonferroni adjustment to further identify the nature of those differences.

Seasonal variability in metal content in *U. australis* was analysed in a similar manner, using a general linear mixed model (GLMM), with area as random effect, year as factor and season as fixed effect, with a gamma distribution and restricted suite of post-hoc multiple contrast using the Least-Square Means (lsmeans) package (Lenth and Hervé 2015) and Bonferroni adjustment.

Seasonal Zn variability in seawater was assessed using a general linear mixed model (GLMM) with a gamma distribution. All GLMM analyses were performed in R with significance of the model parameters determined using alpha = 0.05 (R Core Team 2013).

3.4 RESULTS

- **Spatial metal patterns in spring 2013**

Zinc and lead were detectable in *U. australis* at sample sites in spring 2013. The highest content of Zn and Pb were at Prince of Wales Bay (PWB) and Shag Bay (SB). There was a significant interaction between site location and metal levels in *U. australis* ($p < 0.05$, $F = 252.23$, $df = 21$). Post-hoc comparison suggested that samples from PWB and SB were most similar, but that these locations differed significantly from the other sites ($p < 0.05$). Post-hoc testing also suggested that the spatial distributions of Zn and Pb were similar to each other. The results showed that there was a very marked decrease in metal contents in *U. australis* towards the mouth of the estuary, with evidence of a strong gradient within the middle-upper and middle-lower estuary.

- **Inter-annual variability**

Zinc and Pb content in *U. australis* showed a similar distribution in spring 2013 and 2015. However, the total amounts of each metal differed markedly. In 2013, mean Zn content was 320.7 ± 18.3 $mg \cdot kg^{-1}$ and Pb 76.5 ± 1.4 $mg \cdot kg^{-1}$, whereas in spring 2015 levels of both metals were considerably lower, with Zn at 76.5 ± 1.4 $mg \cdot kg^{-1}$ and Pb at 13.8 ± 2.0 $mg \cdot kg^{-1}$ (Fig. 3.2).

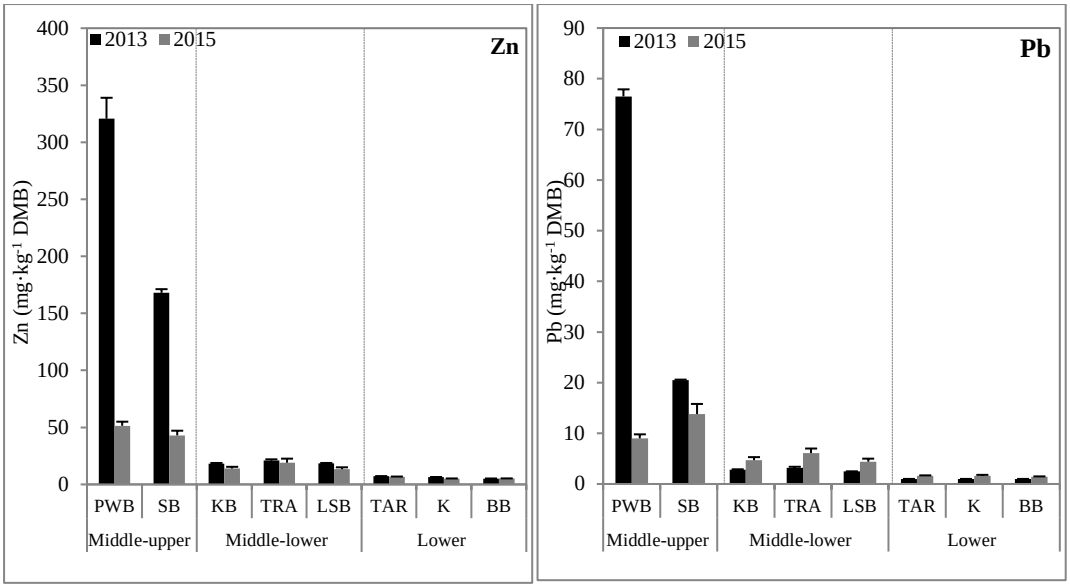


Figure 3.2. Inter-annual zinc (Zn) and lead (Pb) content (mg·kg^{-1} DMB) in *Ulva australis* from sites in the Derwent Estuary in spring 2013 and spring 2015. Metal levels are expressed as mean (± SE). PWB: Princes of Wales Bay, SB: Shag Bay, KB: Kangaroo Bay, TRA: Tranmere, LSB: Lower Sandy Bay, TAR: Taroona, K: Kingston Beach and BB: Blackmans Bay. Note different scales on y-axis.

In both years, Zn content declined along the estuary ($p < 0.05$, $F = 209.9500$, $df = 2$). The spatial gradient showed that from spring 2013 to spring 2015, Zn in *Ulva* decreased 83.9% in the middle-upper estuary (PWB), 9.5% in the middle-lower (TRA), and 5.6% the lower estuary (TAR); this difference was particularly evident in the middle-upper estuary ($p < 0.05$, $F = 137.8373$, $df = 1$) (Fig. 3.2). Post-hoc comparisons indicated that these temporal (long-term) differences were most marked between the more contaminated sites in the middle-upper ($p < 0.05$, t-ratio = 18.071, $df = 37$) and the middle-lower region ($p < 0.05$, t-ratio = 3.155, $df = 37$). The sites from the lower estuary did not differ significantly over time.

Inter-annual Pb variation in *U. australis* also showed a strong spatial gradient ($p < 0.05$, $F = 292.0535$, $df = 2$). As with Zn, Pb content was highest in the middle-upper region (76.5 $mg \cdot kg^{-1}$, PWB), decreased in the middle-lower (6.1 $mg \cdot kg^{-1}$, TRA), and was lowest (1.6 $mg \cdot kg^{-1}$, TAR) in the lower estuary region (Fig. 3.2). Post-hoc comparison indicated that there was a significant decrease in Pb from 2013 to 2015 ($p < 0.05$, t-ratio = 8.763, $df = 37$), which was driven by a strong decrease in the middle-upper estuary levels (88 % in PWB; Fig. 3.2). However, there was an increase in Pb content in 2015 compared to 2013 in both the middle-lower ($p < 0.05$, t-ratio = -4.395, $df = 37$) and lower region of the estuary ($p < 0.05$, t-ratio = -3.200, $df = 37$).

- **Seasonal variability**

There were significant seasonal ($p < 0.05$, $F = 8.076$, $df = 3$) and spatial ($p < 0.05$, $F = 24.3439$, $df = 2$) differences in Zn content in *U. australis*, with samples from the middle-upper estuary changing significantly between seasons (Fig 3.3). Metal content was relatively constant across seasons, with the exception of winter 2015, when the content of Zn increased in the middle-upper estuary. The highest Zn content for all seasons was observed in the middle-upper estuary in spring 2013, with a clear spatial distribution between regions of the estuary at every season.

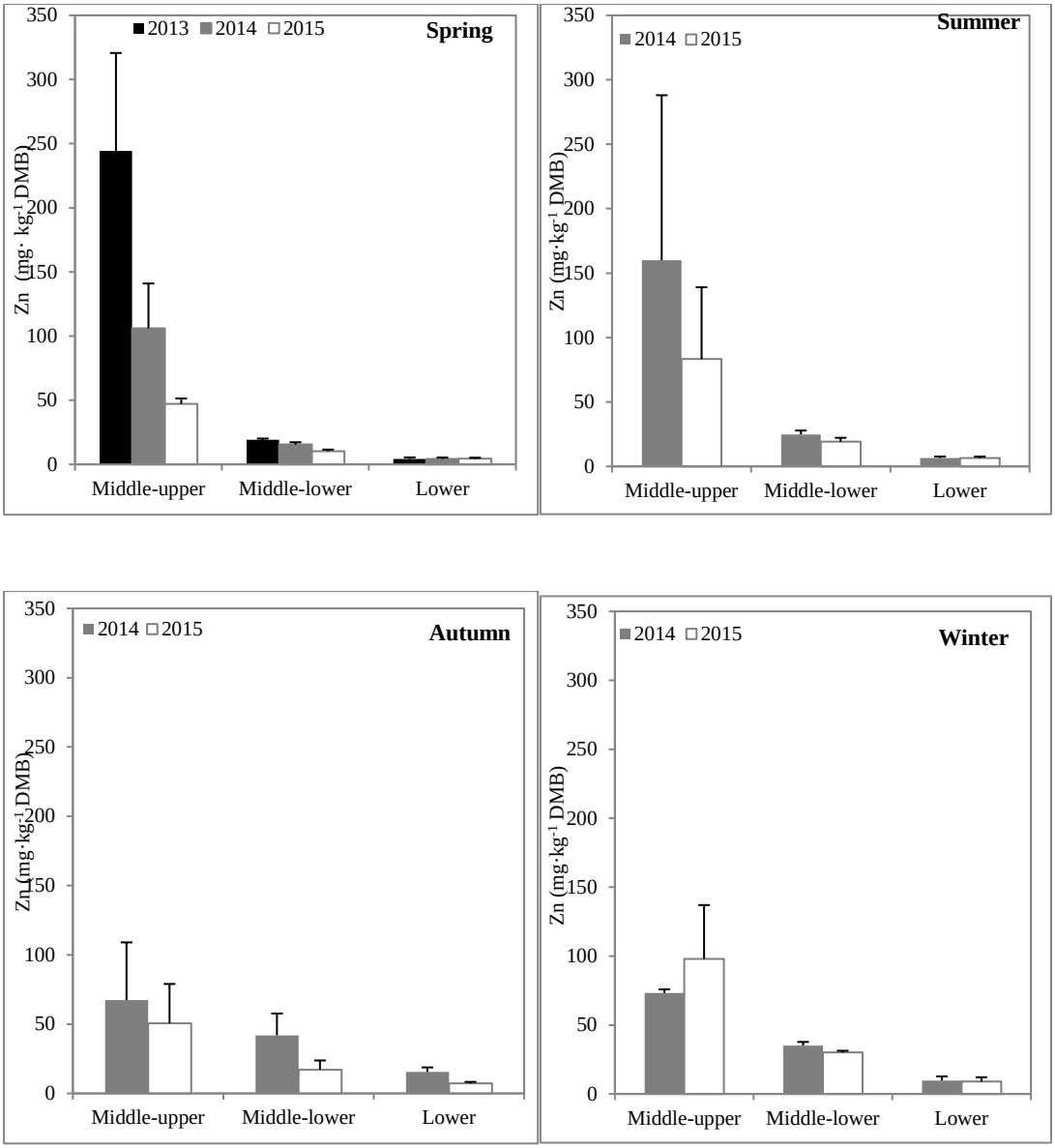


Figure 3.3. Zinc (Zn) content (mg·kg^{-1} DMB) in *Ulva australis* collected from spring 2013, summer, autumn, winter and spring 2014 and 2015, from the three different regions defined in the current study. Metal levels expressed as mean (± SE), at Middle-upper estuary (n = 2), Middle-lower estuary (n = 3), and Lower estuary (n = 3).

There was a strong interaction between season and region ($p < 0.05$, $F = 4.0979$, $df = 6$). post-hoc comparison suggested that this is due to *Ulva* from the middle-upper estuary having a much higher Zn content (244.3 ± 108.0 mg·kg^{-1}) compared to both the middle-lower estuary (42 ± 27.2 mg·kg^{-1}) and the lower estuary (15.5 ± 7.3 mg·kg^{-1}) for all seasons evaluated ($p < 0.05$, $df = 5$). This suggests that seasonal patterns are region-

specific. Zn content in *U. australis* was significantly higher in the middle-upper estuary in summer (160 $mg \cdot kg^{-1}$) and spring (244.3 $mg \cdot kg^{-1}$), than in autumn (67.4 $mg \cdot kg^{-1}$) or winter (98 $mg \cdot kg^{-1}$). In autumn and winter, there were still clear spatial differences in Zn content in *U. australis* between the middle-upper (up to 98 $mg \cdot kg^{-1}$) and the lower estuary (Zn content is <15.5 $mg \cdot kg^{-1}$) ($p < 0.05$, $df = 5$). However, there was no significant difference in Zn levels between the middle-lower and the lower estuary ($p > 0.05$) in any season.

Lead content in *U. australis* collected from the middle-upper estuary decreased from spring to the autumn-winter season (Fig. 3.4). As with Zn, the highest Pb content in *U. australis* was in spring and summer (20 $mg \cdot kg^{-1}$) however, these seasonal differences were not significant ($p > 0.05$). Lead content in *U. australis* was affected by the region of the estuary in which *Ulva* is located ($p < 0.05$, $F = 20.392$, $df = 2$), with the highest Pb content occurring in the middle-upper estuary (20 $mg \cdot kg^{-1}$), and decreasing to the middle-lower (up to 8 $mg \cdot kg^{-1}$) and lower estuary (up to 3.2 $mg \cdot kg^{-1}$) (Fig. 3.4).

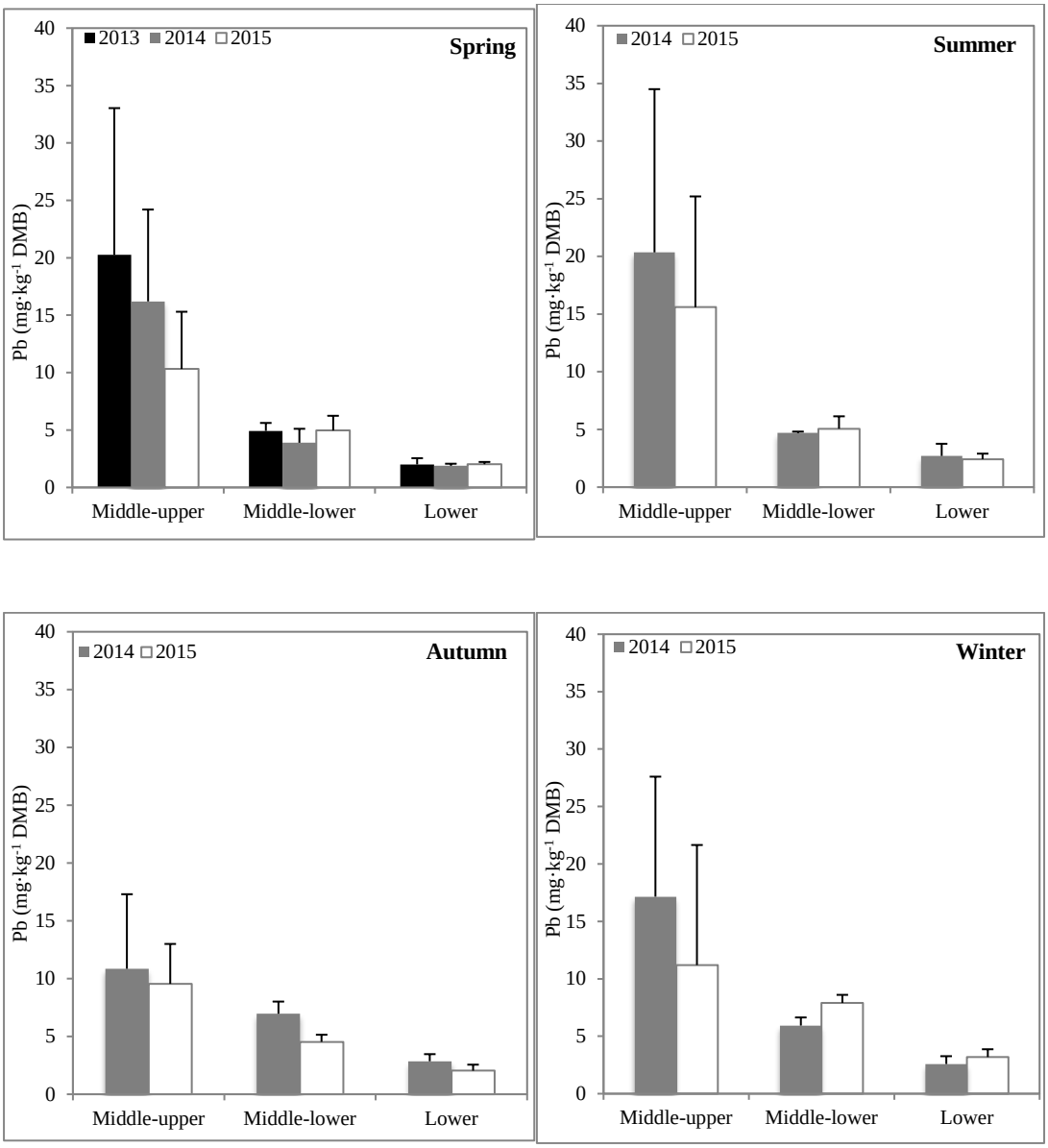


Figure 3.4. Lead (Pb) content (mg·kg^{-1} DMB) in *Ulva australis* collected in spring 2013, summer, autumn, winter and spring 2014 and 2015, from the three regions of the Derwent Estuary. Metal levels expressed as mean (± SE), at Middle-upper estuary (n = 2), Middle-lower estuary (n = 3), and Lower estuary (n = 3).

- **Metal content in seawater**

There was significant spatial variability in Zn concentration in the surface water ($p < 0.05$, $F = 45.623$, $df = 1$), with the concentration in the middle-upper region of the estuary markedly higher than the middle-lower estuary (Fig. 3.5). This difference was consistent over time, with no significant seasonal variability for Zn in surface seawater (Fig. 3.5).

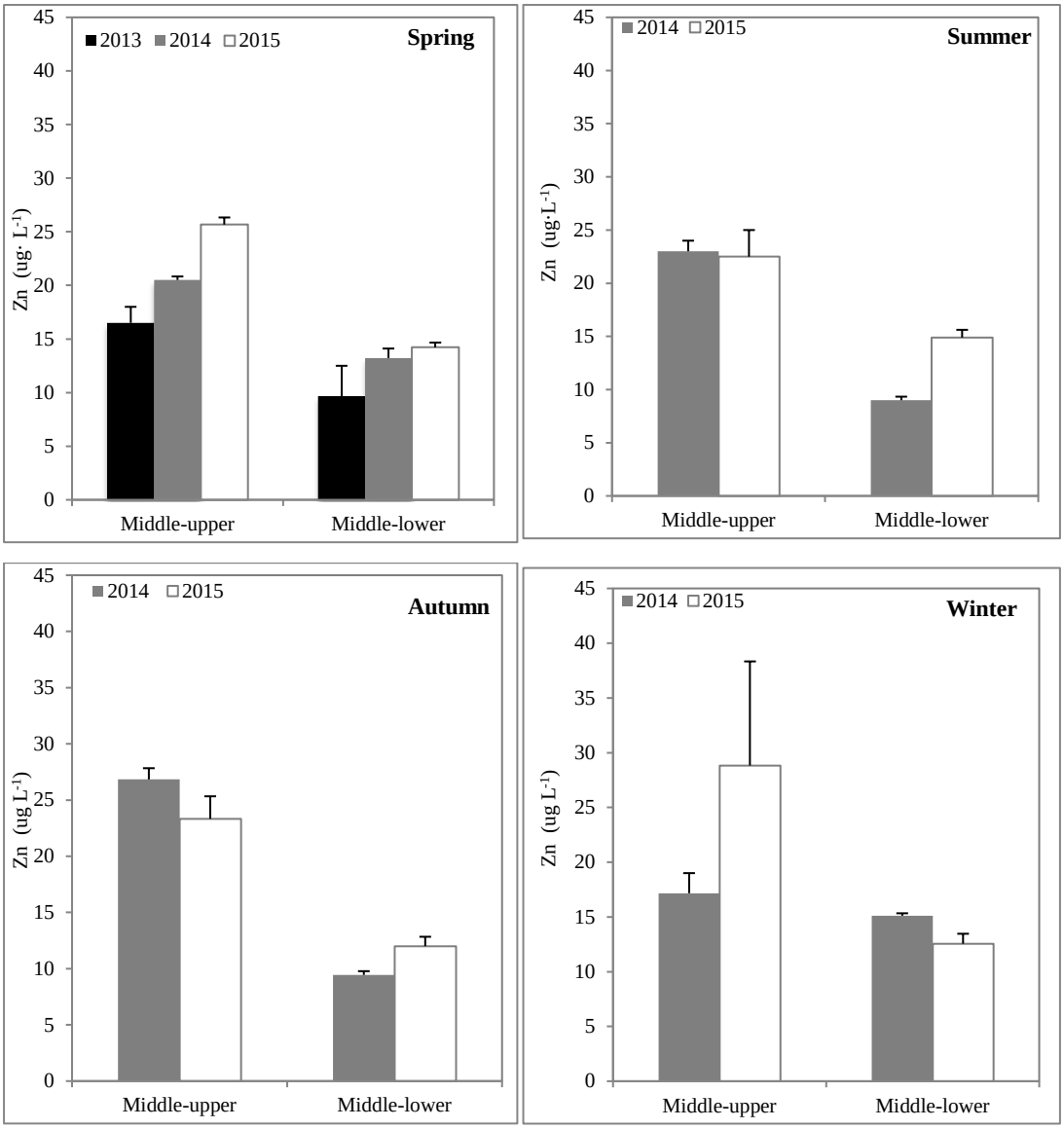


Figure 3.5. Total zinc (Zn) in seawater ($\mu g \cdot L^{-1}$) for samples collected from Middle-upper (n = 2) and Middle-lower estuary (n = 3), in spring 2013, and summer, autumn, winter, spring 2014 and 2015. Metal levels expressed as mean (± SE). Data courtesy of the Derwent Estuary Program (DEP).

3.5 DISCUSSION

This study clearly shows that the metals levels in *Ulva australis* reflected the changes in the environmental loads and as such suggests that *U. australis* could be used as a biological monitoring tool, both in the Derwent Estuary and potentially elsewhere in Australia. The results suggest that *U. australis* would be particularly suitable for biomonitoring Zn levels in the Derwent, the most significant contaminant in the system. However, the findings also highlight the need to consider the potential effects of both inter-annual and seasonal variability when comparing monitoring results and the need to be mindful of any salinity differences, as this can also influence the ability to compare results.

Ulva australis accumulated both zinc and lead, and both metals showed a clear spatial gradient that could be related to environmental loads, which suggests that *U. australis* could be a reliable indicator of metal pollution in the Derwent. The highest algal metal concentrations were found in areas where the environmental loading was also high, i.e. in the middle-upper estuary, at Prince of Wales Bay (PWB) and Shag Bay (SB), closest to the Nyrstar Zn smelter and therefore it is not surprising that the metal content detected in *U. australis* was high at these sites. The distribution of metal contamination in *Ulva* relates well to the patterns of contamination described by the established monitoring of surface waters and sediments (Coughanowr et al. 2015). Consequently, it is reasonable to expect that *U. australis* would provide a realistic and comparable measure of environmental contamination.

Previous research has shown that environmental factors such as salinity can influence *Ulva's* capacity to take up metals (Ho 1990; Malea and Haritonidis 1999a). Laboratory

studies have indicated that metal levels in *Ulva reticulata* increase as salinity declines (Mamboya et al. 2009). This has also been observed in field studies where the influence of rivers and subsequent salinity reductions have been shown to affect metal concentrations in *Ulva* sp. (Villares et al. 2002). There is quite a strong salinity gradient in the Derwent estuary, with salinity being reduced from 34 to 24 ppt in surface waters in the middle-upper estuary, the area where the highest content of Zn were detected in *U. australis*. It is unclear to what extent the salinity might have influenced (increased) the levels in *U. australis* at this location, but the levels observed at these sites are real and have clear biological implications for both the plants themselves and the food web. Consequently, it would be important to consider the potential for salinity effects when incorporating *Ulva* in a graded biomonitoring program, and in particularly how this might affect trend comparisons (temporal or spatial).

In the current study, the temporal differences in metal levels in *U. australis* were clear, with both inter-annual and seasonal differences evident. There are a number of potential sources of temporal variability; changes could be due to differences in the metal loading/ inputs within the system as a whole and/ or changes in the alga's ability to accumulate metals, and both of these could be exacerbated by the timing of sampling. Metal uptake in seaweeds (*Ulva*) can vary as a function of time directly (i.e. the age or condition of the plant) or indirectly (i.e. as a result of seasonal changes in temperature and other environmental conditions). In all instances, the content of Zn and Pb in *U. australis* was highest in the most polluted areas of the estuary, the middle-upper estuary. In these areas, it is likely that the contamination levels are so high that they would likely drown out any subtle seasonal or temporal differences. In the present study, the inter-annual analysis showed that Zn content in *Ulva* varied most between years, whereas Pb content seems to

depend more on the area of the estuary from which the samples were collected, with little temporal variability. This may reflect a combination of both the scale of the Zn contamination in the Derwent and the significant effort from Nystar to mitigate groundwater inputs of Zn into the estuary over the last few years (Coughanowr et al. 2015). The inter-annual assessment demonstrated that the metal content in *U. australis* from the lower estuary was consistently low, with levels similar to that described for other unpolluted systems worldwide (Table 3.1). Consequently, this region would appear to effectively represent the background reference conditions against which the performance of the rest of the system can be gauged in future monitoring. These results may suggest that where the system is highly contaminated, *U. australis* can provide a good indication of the temporal metal variability associated with anthropogenic changes or intervention, but that where contaminations levels are lower perhaps more caution needs to be taken when interpreting results over time and especially in inferring any humans influence to those changes.

Table 3.1. Comparison of means metal content for seaweed (mg·kg^{-1}) and seawater (µg·L^{-1}) both in the current study and elsewhere as reported in the literature for both polluted and unpolluted environments.

Reference	Species	Zn (mg·kg^{-1})	Pb (mg·kg^{-1})
This research [a]	*Ulva australis (middle-upper estuary)* [c]	320	95.4
	Ulva australis (middle-lower estuary) [b]	33.1	21.5
	Ulva australis (lower estuary) [b]	17.8	3.5
Brown et al. (1999)	*Ulva (Enteromorpha) intestinalis* [b]	< 30	-
	Ulva lactuca [b]	< 10	-
Conti and Cecchetti (2003)	*Ulva lactuca* [b]	55	< 3
	Padina pavonica [b]	56	< 5
Guisti (2001)	*Fucus vesiculosus (Holy island)* [b]	17.7	1.1
	Fucus vesiculosus (Roker) [c]	740	7.8
	Fucus vesiculosus (Brand sands) [b]	62.9	3.2
	Fucus vesiculosus (Easignton) [c]	1015.5	12.1
Leal et al. (1997)	*Ulva (Enteromorpha)* [b]	-	< 5
	Porphyra spp. [b]	-	< 3
Ryan et al. (2012)	*Polysiphonia lanosa* [b]	140	< 5
	Ascophylum nodosum [b]	80	< 5
	Fucus vesiculosus [b]	25	< 5
	Ulva sp. [b]	10	< 5
Say et al. (1990)	*Ulva* spp *(Enteromorpha)* [c]	> 150	> 60
Stengel et al. (2004)	*Ulva (Enteromorpha) intestinalis* [b]	25.61	-
	Ulva lactuca [b]	25.61	-
	Cladophora rupestris [b]	25.61	-
Author	**Source**	**Zn**	**Pb**
DEP [d]	Surface water *(middle-upper estuary)* [c] (µg·L^{-1})	22.7 ± 1.4	-
	Surface *(middle-lower estuary)* [b] (µg·L^{-1})	12.2 ± 0.8	-
	Surface *(lower estuary)* [b] (µg·L^{-1})	-	-
	Sediment *(middle-upper estuary)* [c] (mg·kg^{-1})	6 440 - 14 600	1 090 - 1 880
	Sediment *(middle-lower estuary)* [b] (mg·kg^{-1})	705 – 2 190	227 - 341
	Sediment *(lower estuary)* [b] (mg·kg^{-1})	46 - 989	27 - 329
Conti and Cecchetti (2003)	Seawater [b] (µg·L^{-1})	10.4 ± 0.12	0.003 ± 0.06
	Seawater [b] (µg·L^{-1})	-	< 4
Ryan et al. (2012)	Seawater [b] (µg·L^{-1})	1.0 ± 0.0	0.1 ± 0.0

[a] Maximum values observed in this research; [b] Unpolluted environment; [c] Polluted environment; [d] Data courtesy of Derwent Estuary Program (DEP)

Temporal variability in metal contamination in seaweeds species can also be related to biological factors such as the growth strategy (annual/ perennial) or to the morphology of the particular species (Stengel et al. 2004). Many studies have shown differences between species, *Ulva linza* (Formerly *Enteromorpha linza*) has been shown to take up metals to a greater extent in spring and summer (Haritonidis and Nikolaidis 1990), but *U. lactuca* was

found to have the highest Zn content in winter (Brown et al. 1999), Villares et al. (2002) studied two related *Ulva* species (*U. rigida* and *U. intestinalis*) and found that metal content was greatest in autumn/winter, and Haritonidis and Malea (1999) observed maximum metal content in *U. rigida* in autumn. These differences are likely due to changes in plant metabolism, which in itself can be confounded by temperature and a range of other environmental variables. In this investigation, Zn content in *U. australis* was greater in spring and summer than other times of the year. This is the time when growth/ metabolic rate was highest (in summer) and/ or when the plants were reproductive (in spring). Consequently, these physiological factors may need to be taken into account when comparing temporal trends in metal load, and illustrating the importance of being mindful of the potential extent of temporal variability both within a specific system (contamination and background environmental conditions), within the biomonitoring species (ecology), and the need to be aware of this source of variation when comparing and analysing information for monitoring programs.

Another important aspect that needs to be considered when selecting macroalgae for biomonitoring is plant morphology, as the region sampled may also influence the measure of contamination as metals may be taken up differently in different sections of the thallus (Stengel et al. 2004; Ho 1990). Fortunately, this does not apply in the case of *Ulva*, as the "sheet-like" morphology allows metals to accumulate across the whole thallus. Therefore, *Ulva* is morphologically a good genus to be used as a biomonitor, as collection of any part of the plant should offers a consistent assessment of the levels of metals in the system.

The ambient water quality monitoring program managed by the DEP demonstrated that, in the Derwent, Zn was the predominant contaminant, and a suitable proxy for most other

heavy metals contaminants (Cu, As and Pb) (Coughanowr et al. 2015). Zinc is consistently detected at high concentrations in the water column, sediments, fish and shellfish. Consequently, evaluating Zn levels in a biomonitor species from the Derwent should provide a good indication of the broader metal contamination in this system. In this instance, using a biomonitoring species such as *Ulva* is particularly valuable, as it can provide an integrated response of the Zn contamination throughout the estuary over time (the growing period of the plant), whilst samples from the water column will only ever provide a snapshot of the concentration at the time of sampling. The results showed that although *U. australis* broadly reflected a similar contamination gradient and pattern to that observed in the water column/ sediment sampling the levels were quite different, and the metals in *Ulva* reflect a more directly bioavailable contaminant source, which has been missing in monitoring to date. Monitoring of *U. australis* could provide important information on changes in this bioavailable component over time, as a result of remediation/ deterioration activities and therefore, it is suggested that *U. australis* be incorporated into future biomonitoring programs.

Finally, one of the key advantage for applying *U. australis* as a biomonitoring tool is the fact that this species in both locally abundant and ubiquitous throughout the estuary. Previous studies have suggested that local species are generally the most suitable for biomonitoring (Rainbow 2006; Phillips and Rainbow 1994), and that cosmopolitan, widely distributed (O'Leary and Breen 1997) or abundant (Amado Filho et al. 1999) species are to be preferred. Being an Ulvacean with a highly cosmopolitan distribution (northern- and southern-hemisphere), *U. australis* satisfies many of the key pre-requisites proposed by those earlier studies, and may be equally useful for biomonitoring in other coastal and estuarine systems around Australia and potentially could be compared with

other worldwide *Ulva* species. But, most importantly, it reflects the changing metals loads in the system.

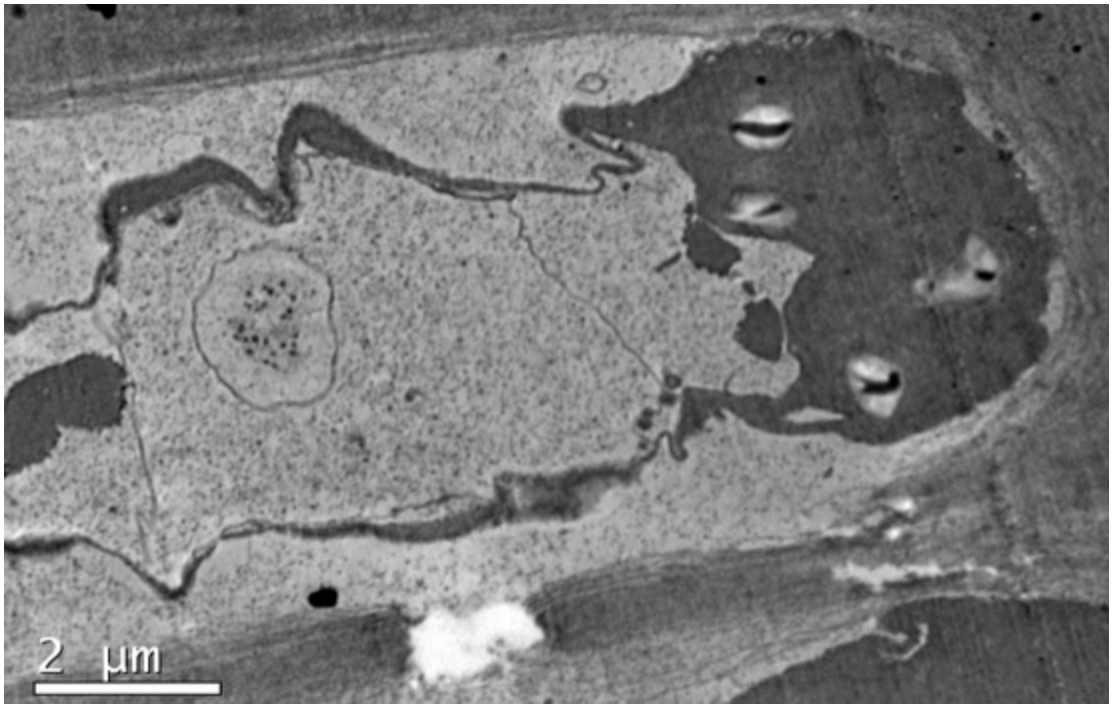
Currently seaweeds are not included in the Derwent Estuary monitoring program. Adopting *U. australis* as an indicator in this program, in conjunction with seawater and sediment monitoring, would provide another line of evidence with which to assess changes (improvement/ deterioration) in the condition of this metal-impacted system. Including *U. australis* would give the monitoring program a better understanding of the "biological condition" of the system, providing an integrated assessment of the conditions at each location and time.

3.6 CONCLUSIONS

There was a strong spatial gradient of metal contamination within the estuary, with the middle-upper estuary having an extremely high level of Zn pollution. This spatial variability was clearly reflected in *Ulva australis*. There was evidence of seasonal differences in the metal content in *Ulva*, with the highest levels observed in spring and summer. Environmental conditions, particularly salinity, may affect results and therefore should be treated with caution where there are marked changes in salinity. Biological factors (e.g. growth or morphology) also affected metal content in *U. australis* with levels of contamination being influenced by the key biological processes/ phenology. However, it is clear that *U. australis* can play a valuable role as biomonitoing species, and can help improve our understanding of the potential risks and changes (improvements and deteriorations) in metal contaminated systems.

CHAPTER 4

Fine-tuning transmission electron microscopy methods to evaluate the cellular architecture of Ulvacean seaweeds (Chlorophyta)



Preface:
This investigation arises from the necessity to analyse the ultrastructure of *Ulva australis* using Transmission Electron Microscopy (TEM). We observed that there were no comparable methods to use for green macroalgae species. We evaluated five different TEM protocols in *Ulva lactuca*, to standardise TEM methods for Ulvacean. Our results showed that the standardisation of a new protocol will ensure that the ultrastructure of Ulvacean seaweeds can be clearly ascertained in future TEM analysis involving heavy metals.

This work has been published in Micron refereed journal and it has been slightly modified for thesis format. DOI: 10.1016/j.micron.2017.02.003.

4.1 HIGHLIGHTS

- This study proposes a novel and highly effective protocol for ultrastructure assessment of Ulvacean seaweeds
- A combination of primary fixatives, glutaraldehyde and paraformaldehyde, gave the best fixation approach for differentiation of the internal components of *Ulva*,
- Ethanol provides the optimal outcome in the dehydration process prior to Transmission Electron Microscopy for Ulvacean seaweeds.

4.2 ABSTRACT

Chemical fixation is a critical step in the analysis of the ultrastructure of seaweeds because the wrong approach can compromise the ability to distinguish fine-scale cellular composition. Fixation agents, fixation time and type of tissue are important factors to consider for transmission electron microscopy (TEM), and not every protocol is suitable for all cell types. We evaluated a range of fixation agents, post-fixation time and dehydration solutions to determine a TEM protocol for seaweeds in the Family Ulvaceae. We assessed *Ulva lactuca* using 5 protocols. The level of preservation obtained differed markedly between fixation methods. The best result was obtained by fixing the sample with 2.5% glutaraldehyde, 0.05 M sodium cacodylate buffer and 2% paraformaldehyde overnight, and 8 h post-fixation in 1% in osmium tetroxide 1%. This approach and fixation time ensured that the membranes, especially the thylakoid membranes of chloroplasts, remained intact. Ethanol is recommended for dehydration as the use of acetone for dehydration resulted in the collapse of cellular membranes. This new protocol will ensure the ultrastructure of Ulvacean seaweeds can be clearly ascertained in the future.

Key words: Microscopy; Seaweed; TEM protocol; Ultrastructure; *Ulva lactuca*.

4.3 INTRODUCTION

Chemical fixation is one of the most important stages in evaluating seaweed ultrastructure. Fixation is the first step for microscopic analysis. A poorly preserved specimen could be unusable for any research. However, not every protocol suits all types of cells. For transmission electron microscopy (TEM), it is important to consider basic fixation procedures including reagent and fixation time and type of tissue to be assessed. Different chemical reagents are used to preserve the structure of the living tissue and stabilize the internal cellular structure (Bozzola and Russell 1999; Shah et al. 2014). The standard fixation process involves placing the material in a fixation solution for a couple of hours. This solution varies depending on tissue and sample objective, as well as reagent concentrations or pH (Hayat 2000). The main fixation reagents used in microscopy, are glutaraldehyde (GTA) and paraformaldehyde (PFA), both aldehydes with each having different tissue penetration rates. Glutaraldehyde works quickly to stabilize the structure however, the penetration rate varies depending on the tissue sampled and is generally slow therefore, a very thin section is required for rapid penetration (1 mm tissue thickness) (Bozzola and Russell 1999). In contrast, paraformaldehyde rapidly fixes the internal cell components as it can penetrate the tissue quickly (Karnovsky 1965; Kiernan 2000). Consequently, a combination of glutaraldehyde and paraformaldehyde should provide for excellent fixation under most circumstances (Karnovsky 1965).

Several fixation protocols have been developed for particular tissues or cells. However, there are no general TEM methods for seaweeds, because the internal structure of red, brown and green seaweeds varies. While TEM methods for red and green seaweeds are similar, specific TEM methods have been adapted for brown species to preserve phenolic compounds. Specific modification includes incorporation of caffeine for stabilising the

phenol in the vacuole and calcium chloride ($CaCl_2$) for the preservation of phospholipids in tissues (Rover et al. 2015; Polo et al. 2014; Holzinger et al. 2011a). Targeted protocols have also been developed for early life-stages in brown species because it was found that TEM methods for adult thalli were inappropriate for spores (Steinhoff et al. 2008). In red seaweed, the same fixation methods are applied to early life-stages as adult thalli (Simioni et al. 2014). Protocol standardization is essential, because different approaches can create significant distinctions in cell ultrastructures (Mogi et al. 2008); poor fixation can result in key structures or membranes being obscured, and the dehydration process can cause tissue retraction.

Ensuring well-preserved tissue samples is critical for microscope analysis. Consequently, there is a need to develop appropriate TEM methods for each seaweed group. A large variety of different TEM methods, including different reagent concentrations and timing of fixation, have been used to investigate ultrastructure in green seaweeds (Holzinger et al. 2010; Karsten and Holzinger 2012; Murakami and Packer 1970; Roleda et al. 2010; Schiavon et al. 2012; Vecchia et al. 2012; Holzinger and Karsten 2013). The aim of this study is to determine an appropriate fixation method for TEM for a specific group of green seaweeds – Ulvacean – using the common green seaweed *Ulva lactuca*.

4.4 METHODS

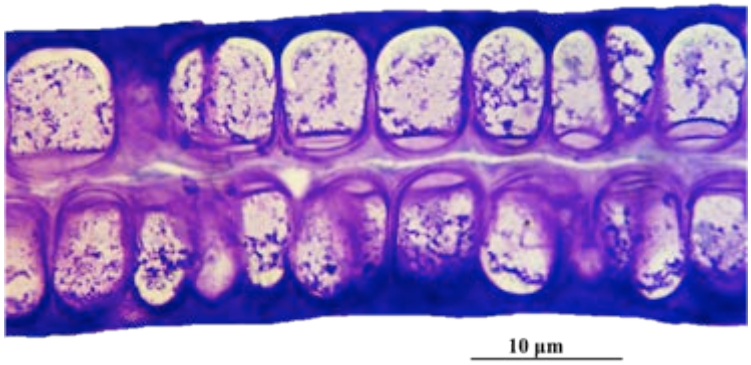
To develop an appropriate TEM fixation method for Ulvacean seaweeds (Chlorophyta), examining approaches for a wide range of species, morphology, and life-stages, for green (Chlorophyta), red (Rhodophyta) and brown (Ochrophyta) seaweeds (Table 4.1). We compared the more common methods used for red and brown seaweeds (Method A), and a variety of methods for green seaweeds (Methods B-E). Summaries of these methods are presented in Table 4.1.

- **Samples collection and experiment set up**

Ulva lactuca Linnaeus, C. (1753) thalli (Fig. 4.1) were collected from the shoreline at Ponta Das Canas, Florianopolis, Santa Catarina, Brasil, during low tide on the 7th of May 2015. Samples were transported to the laboratory in plastic bags in an insulated container. In the laboratory, samples were cleaned with filtered seawater to remove sand and epiphytes, and then thalli were cut into 1 mm width portions and fixed in individual vials. For all samples, excisions were taken from the external outer edge of the thallus. Transverse sections of thalli species were stained with toluidine blue and observed with light microscopy (40x) in an Epifluorescent microscope (Olympus BX 41), equipped with Image Q Capture Pro 5.1 software (Qimaging Corporation, Austin, TX, USA), to identify cell organization (Fig. 4.2). Specimens, were compared with *Ulva* spp. from the Herbarium of Department of Botany, Federal University of Santa Catarina to verify species.

Figure 4.1. *Ulva lactuca* specimen collected from Ponta Das Canas, Florianopolis, Santa Catarina, Brasil. (Image taken with Nikon Coolpix S30).

Figure 4.2. Transversal sections of *Ulva lactuca* stained with toluidine blue, observed with light microscopy (40x), demonstrating typical cell configuration for ulvacean, with two single layers of cells.



- **Transmission Electron Microscopy (TEM)**

The samples were treated with 5 different fixation and post-fixation methods (Table 4.2), the specific methods are described below.

Method A:

Fixation in 2.5% glutaraldehyde (GTA), 0.1 M sodium cacodylate buffer (pH 7.2) and 0.2 M sucrose, overnight at 4°C. Post-fixation was for 4 h in 2% osmium tetroxide (OT) and dehydrated using an acetone succession prior to embeddeding in Spurr's resin to produce a solid block for sectioning.

Method B:

Fixation in 2.5% GTA and 0.1 M sodium cacodylate buffer (pH 7.2), for 4 h at 4 °C. Samples were the post-fixed with 1% OT overnight and dehydrated later with ascendant ethanol succession before being embedded in Spurr's resin.

Method C:

Fixation in 2.5% GTA and 0.1 M sodium cacodylate buffer (pH 7.2), overnight at 4 °C. Samples were post-fixed with 1% OT for 8 h followed by an ascending ethanol succession before being embedded in Spurr's resin.

Method D:

Fixation in 2.5% GTA, 0.05 M sodium cacodylate buffer (pH 7.2) and 2% paraformaldehyde (PFA), for 4 h at 4 °C. Samples were post-fixed overnight in 1% OT followed by an ascending ethanol succession before being embedded in Spurr's resin.

Method E:

Fixation in 2.5% GTA, 0.05 M sodium cacodylate buffer (pH 7.2) and 2% PFA overnight at 4 °C. Samples were post-fixed in 1% OT for 8 h, followed by a dehydration in an ethanol series before embedded in Spurr's resin.

For all methods above, the Spurr's resin (Low viscosity embedding media, Electron Microscopy Sciences, USA) polymerization time was 24 h at 70 °C. Spurr's resin blocks were firstly cut to 700 nm in an ultramicrotome to localize samples. These sections were then cut into ultrathin sections (60 nm) in an ultramicrotome (Leica EM UC7). The ultrathin sections were placed into a copper grid for contrasting; samples were placed in

5% aqueous uranyl acetate for 10 min in darkness followed by 10 min in 1% lead citrate, according to the methods described by Reynolds (1963). All samples were examined and photographed using a TEM JEM-1011 (JEOL Ltda., Tokyo, Japan, at 80 kV). Cutting and contrasting methods were performed at the Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brasil (LCME-UFSC).

4.5 RESULTS

The five fixation protocols resulted in visible differences in the TEM outcome for *Ulva* tissue (Table 4.3). Fixation method A resulted in a poorly fixed specimen, because the cell membrane collapsed during contrasting. Small portions of tissue were attached to the copper grid as demonstrated by the rupture of the tissues and internal components of the cell (Fig. 4.3a and 4.3c). Furthermore, the internal organelles were clearly not adequately preserved as demonstrated by the lack of definition in the cellulose microfibrils of the cell wall, the thylakoids of the chloroplasts (Fig. 4.3b), the starch grains and the pyrenoid (Fig. 4.3c and 4.3d).

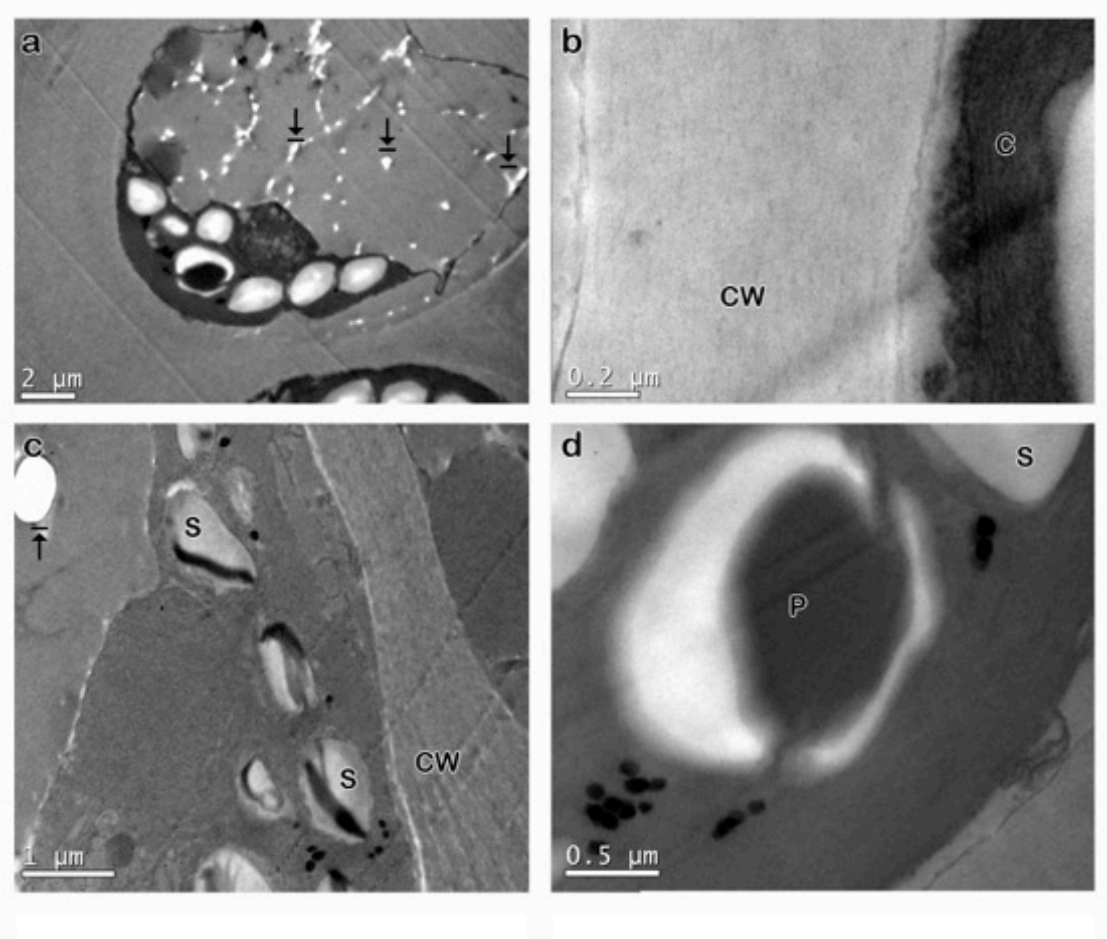


Figure 4.3. Transmission electron microscopy (TEM) micrographs of *Ulva lactuca* cell fixed by Method A. a) general vision of a single cell, arrows indicating rupture of internal cell tissue b) detail of cell wall and chloroplast, c) detail of starch grains, cell wall and collapse of membrane, and d) detail of the pyrenoid surrounded a starch grain. C: chloroplast, CW: cell wall, S: starch granules, P: pyrenoid

Fixation method B was also sub-optimal with the thylakoids membranes of the chloroplast being difficult to identify (Fig. 4.4b). However, the cellulose microfibrils of the cell wall were better preserved than in method A (Fig 4.4b), and the lipids (Fig. 4.4c) and the pyrenoid (Fig. 4.4d) were well preserved.

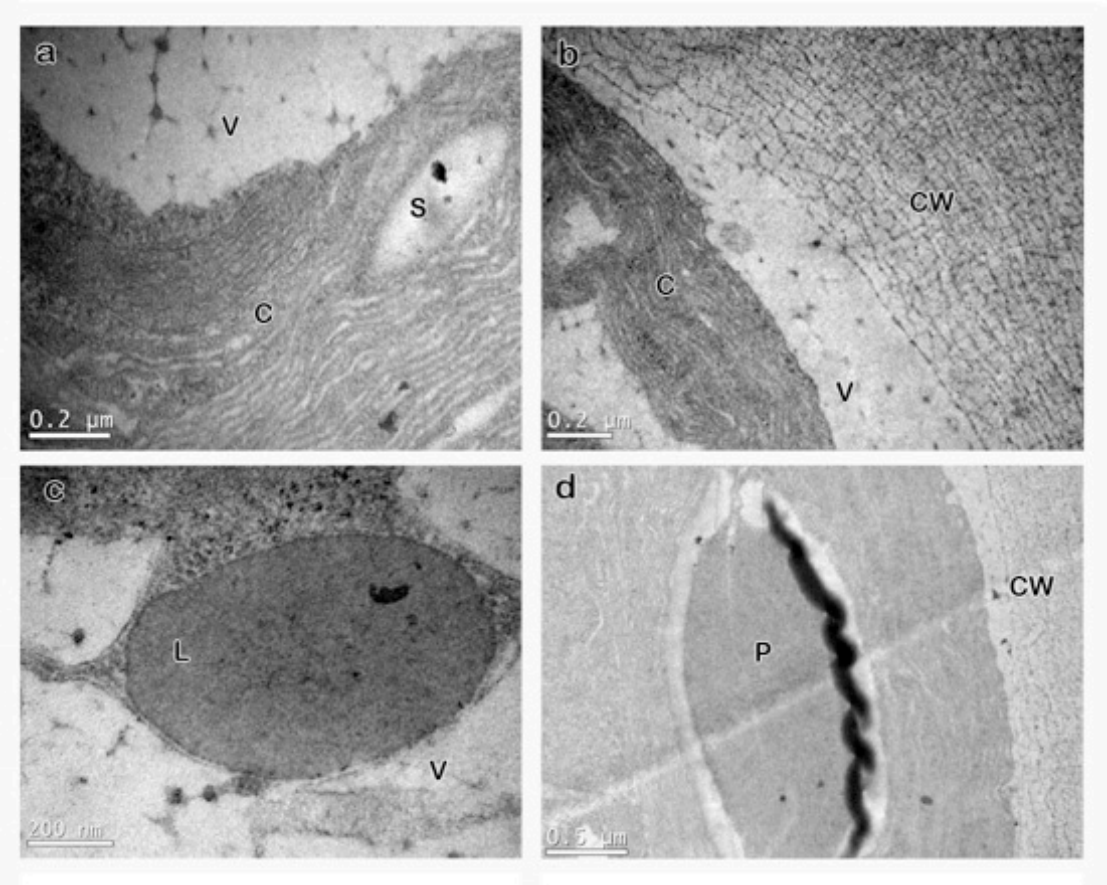


Figure 4.4. Transmission electron microscopy (TEM) micrographs of *Ulva lactuca* ultrastructure fixated by method B. a) detail of cell internal organelles as chloroplast, vacuole and starch grains, b) detail of cellulose microfibrils of cell wall and thylakoid of the chloroplast divided by vacuole, c) details of lipid drop next to the vacuole and d) detail of the pyrenoid surrounded by starch grains next to cell wall not well defined. C: chloroplast, CW: cell wall, S: starch granules, L: lipid, P: pyrenoid, V: vacuole.

Method C resulted in the internal cell organelles being poorly preserved. In this case the overnight fixation would appear to have altered the preservation of the thylakoid membranes of the chloroplast (Fig. 4.5a), and the starch grains were not well defined (Fig. 4.5b and 4.5d). However, the cellulose fibrils of the cell wall showed better preservation than method B (Fig. 4.5a, 4.5c and 4.5d).

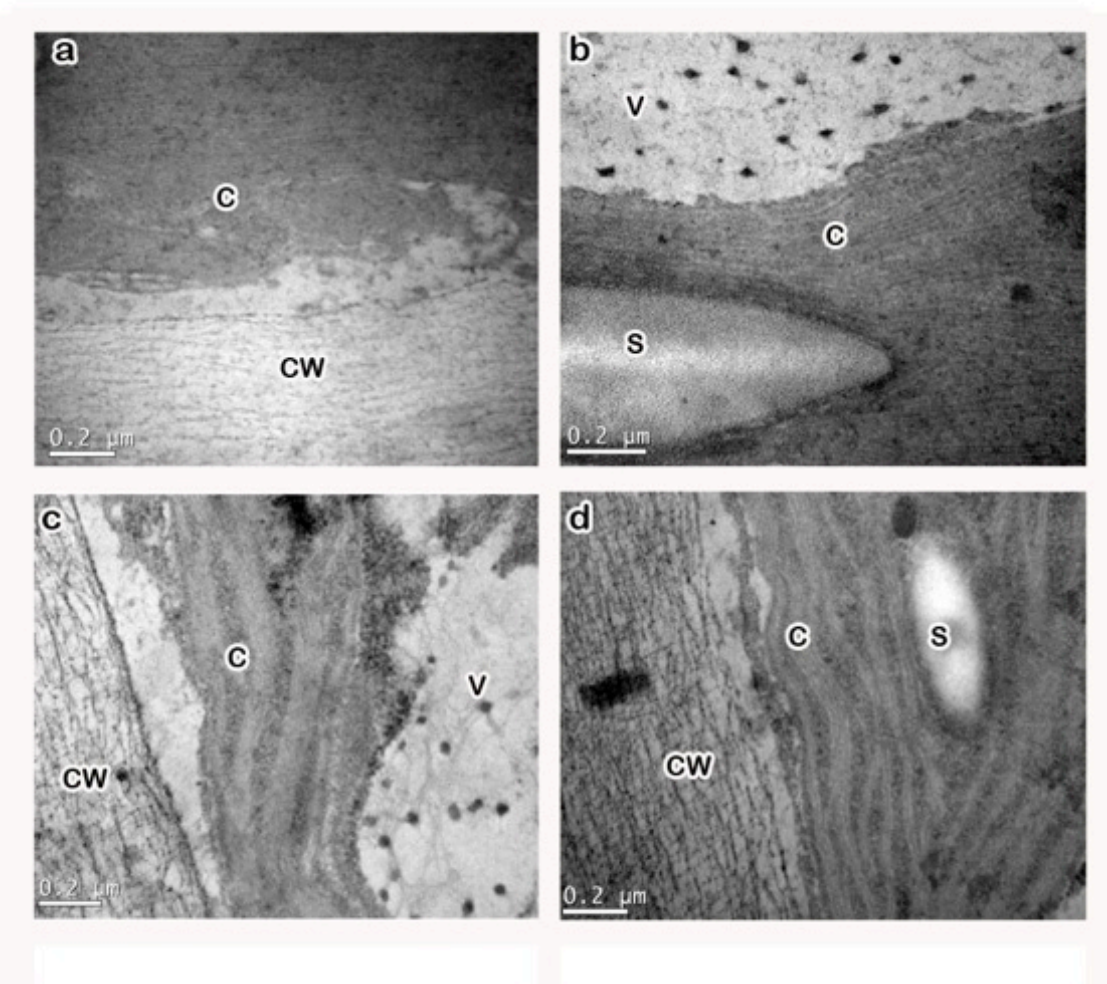


Figure 4.5. Transmission electron microscopy (TEM) micrographs of *Ulva lactuca* ultrastructure fixed by method C. a) detail of cell wall and chloroplast not clear, b) details of thylakoids membrane of the chloroplast surrounding a starch grain, and delimitation of the vacuole c) detail of cellulose fibrils of cell wall, thylakoids and vacuole not well defined, d) detail of cellulose fibrils of cell wall delimitation with chloroplast, and starch grains. C: chloroplast, CW: cell wall, S: starch granules, V: vacuole.

Fixation method D showed that 4 hours of fixation time using GTA, buffer and 2% of PFA did not preserve the internal cell components of *U. lactuca* appropriately. Some components such as the starch grains (Fig. 4.6a) and the thylakoid membranes of the chloroplast were not well defined (Fig. 4.6a, 4.6b and 4.6d). However, the lipid body could be clearly identified (Fig. 4.6c), and the mitochondria were relatively well preserved (Fig. 4.6d).

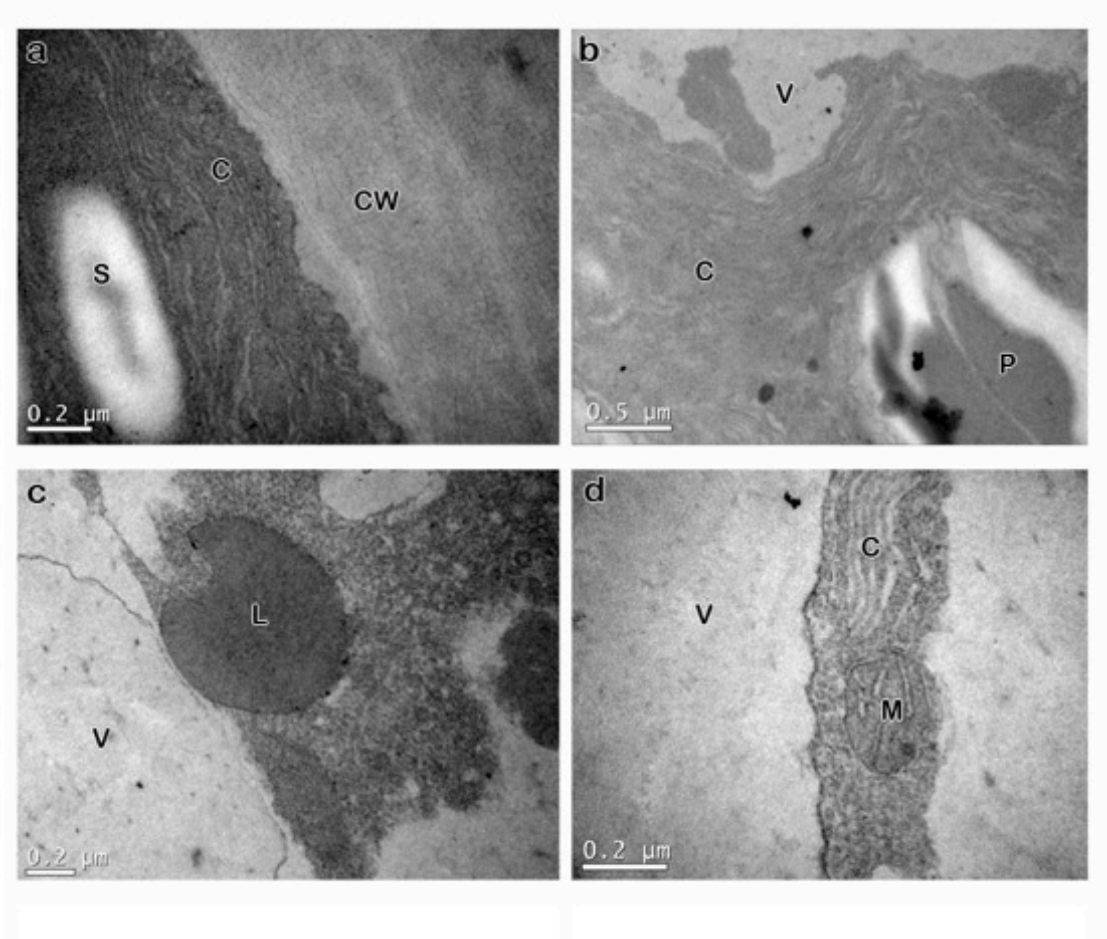


Figure 4.6. Transmission electron microscopy (TEM) micrographs of *Ulva lactuca* internal ultrastructure fixed by Method D. a) starch grains, thylakoids of chloroplast and cell wall cellulose fibrils not well definited, b) chloroplast, vacuole and pyrenoid not clear, c) vacuole and lipid drop d) vacuole, chloroplast and mitochondria not clear. C: chloroplast, CW: cell wall, L: lipid, M: mitochondria, P: pyrenoid, S: starch granules, V: vacuole.

For method E, the primary fixation time was extended to overnight, in GTA, buffer and 2% of PFA. This method showed some improvements on all the other approaches; with generally better preservation of the organelles. The organization of the cellulose fibrils of the cell wall was clearly visible (Fig. 4.7a); thylakoids were well defined and clearly organized into groups of 2-6 (Fig. 4.7b). The other internal components such as lipid drops and the pyrenoid, were also well defined (Fig. 4.7c and 4.7d).

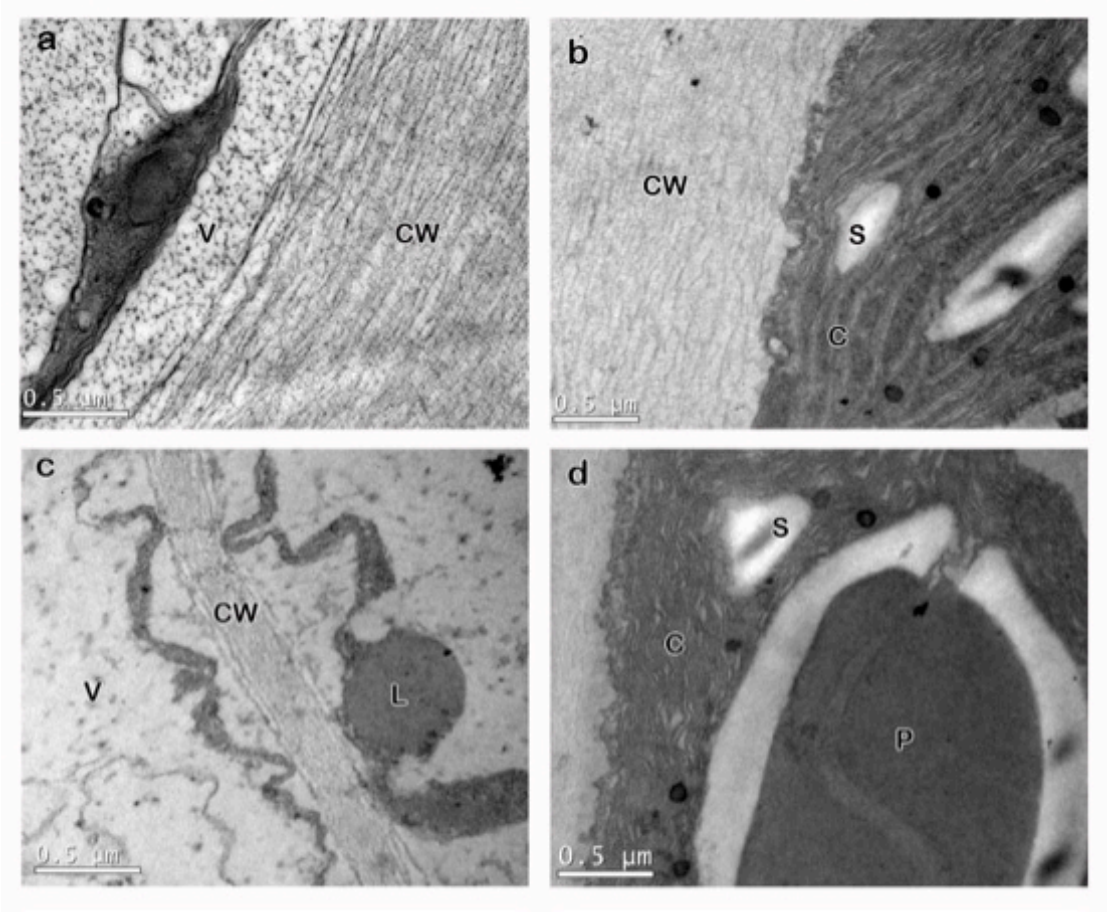


Figure 4.7. Transmission electron microscopy (TEM) micrographs of *Ulva lactuca* internal architecture fixed by method E. a) details of internal composition of vacuole and cellulose fibrils of chloroplast are defined, b) cell wall, groups of chloroplast's thylakoid membranes surrounding starch grains that are well defined, c) cell wall dividing two cells, vacuole and a lipid drop well demarcated, and d) chloroplast, starch grain and pyrenoid well preserved. C: chloroplast, CW: cell wall, L: lipid, S: starch granules, P: pyrenoid, V: vacuole.

4.6 DISCUSSION

Two of the five fixation methods (D and E) applied in *Ulva lactuca* for TEM resulted in well-defined internal architecture. Combining the two primary fixatives, GTA and PFA, as well as the incorporation of ethanol for dehydration produced adequate fixation and structural stabilization visible in the TEM.

The combination of GTA and PFA for fixation has been recommended in previous studies as a means to obtain rapid penetration in the tissue and structural stabilization (Kiernan 2000; Karnovsky 1965). In land plants, the combination of GTA and osmium tetroxide (OT) has been shown to improve tissue fixation (Bozzola and Russell 1999; Sabatini et al. 1963). Osmium tetroxide is a secondary fixative, with a slow fixation rate, but a relatively rapid penetration of osmium into the tissue (occurs after one hour). Prolonged post-fixation times can produce future damage in some internal cell components, when the dehydration will happen (Bozzola and Russell 1999).

The results of the current study suggest that an optimal post-fixation time would be 8 h for the Ulvacean tissue (Method E). This is consistent with the post-fixation times previously reported in the literature for Chlorophyta (green seaweeds), which varied from 2 h up to overnight (~12 h), while for Rhodophyta (red seaweeds) post-fixation time was 4 h and in Ochrophyta (brown seaweeds) varies between 1 to 16 h (Table 4.1).

The fixative solution tested in method A failed primarily as a result of the use of acetone in the dehydration process, which caused the collapse of the cell membrane in *Ulva* and resulted in a poor quality sample for TEM analysis. Dehydration is the process where water inside the cell is replaced with an organic solvent. Ethanol has been considered a more suitable dehydration agent than acetone because acetone absorbs water from the atmosphere, which in some cases can cause loss of lipids (Bozzola and Russell 1999). The strong dehydrating effect of acetone seems to be detrimental to the delicate nature of the internal membrane of *Ulva*. Ethanol has previously has been shown to be more effective in the dehydration process for *Ulva* sp. and the red species *Porphyra* sp. (Murakami and Packer 1970). Method A, is more commonly used for red and brown seaweeds, because

the thicker tissue is more suitable for dehydration with acetone (Simioni et al. 2014; Bouzon et al. 2012; Bouzon et al. 2011; Schmidt et al. 2010; Schmidt et al. 2009b; Rover et al. 2015; Maier 1997). Method A also includes sucrose in its recipe, and commonly this reagent is incorporated to assist with the membrane permeabilization for better penetration of the fixative solution. However, it is believed that sucrose does not interfered on the membrane collapse.

GTA (Methods B and C), is the most common primary fixative for green seaweeds, however, fixation timing usually varies between 1 and 2 h (Holzinger and Karsten 2013; Karsten and Holzinger 2012; Holzinger et al. 2011b; Holzinger et al. 2010; Holzinger et al. 2006; Roleda et al. 2010; Schiavon et al. 2012) rather than the 4 h treatment used in this instance. Only one of the ten publications evaluated for green species in this investigation used the combination of the two primary fixatives, GTA and PFA in chlorophytes (Gasulla et al. 2013).

In this research, the optimum method for fixation of *U. lactuca* (Method E), differs from that proposed for other Ulvacean seaweeds in the literature (Table 4.1). We observed that the addition of 2% PFA as a primary fixative followed by 8 h of post-fixation considerably improved the quality of the sample. Two other methods (method B and C) for fixation of *Ulva* sp. have been described in the literature however, these approaches do not include PFA, and the post-fixation times in the OT were less than 2 h (Schiavon et al. 2012; Murakami and Packer 1970). Murakami and Packer (1970) observed irregular chloroplast shape and shrinkage of the whole chloroplast, which could be related to fixation process.

The appropriate concentration of buffer in tissue fixation is another relevant factor that could affect tissue preservation. Method E, used sodium cacodylate as a buffer at 0.05 M, and this concentration was used in green algae in most of the publications reviewed (Table 4.1). The primary purpose of this buffer is to regulate the osmolarity of the buffering system, because any changes in osmolarity will result in shrinkage or swelling of the tissue (Bozzola and Russell 1999). The results of this study indicate that this concentration is the most appropriate for Ulvacean seaweeds. Bozzola and Russell (1999) established that some tissues show little difference when they are preserved in buffer systems at molarities ranging from 0.05 to 0.2, while other tissues are very sensitive to buffer osmolarity.

The findings of this study show that for *U. lactuca*, the combination of GTA (2.5%), PFA (2%) and buffer 0.05 M was the best fixative solution. Previous studies have similarly shown that the fixation process can be more effective when tailored to specific algae. For example, in the green filamentous species *Zygogonium ericetorum*, tissue preservation was optimized when GTA was reduce from 2.5 to 1.25% and buffer concentration from 0.01 M to 0.05 M (Holzinger et al. 2010). However, this does mean that TEM protocols for green seaweeds (and Ulvaceans in particular) cannot be compared with brown species, which tend to have a more standard fixation protocol that includes calcium chloride ($CaCl_2$) and caffeine (Polo et al. 2014).

TEM protocols can also differ between adult plants and early life-stages; brown algae consistently differ (Table 4.1), but less often in red species (Simioni et al. 2014; Schmidt et al. 2009b; Bouzon et al. 2011). Interestingly, there was only one example in the literature reviewed where a different approach was used for early life-stages in Ulvacean

(*Ulva compressa*) (Mogi et al. 2008), where they used $CaCl_2$ in their protocol for visualising mating structure. In this investigation, TEM analysis of early life-stages of *U. lactuca* was not covered, as it was out of the scope of this research. Therefore, future investigation in this area is needed.

This study reviewed those TEM approaches most likely to be suitable for fixation of *U. lactuca*, and identified an optimum strategy. Providing a standardised method for TEM that can be reliably used to ensure clear definition of the cellular ultrastructure and which will facilitate accurate comparison between studies into the future.

4.7 CONCLUSIONS

A primary fixative formula of 2.5% GTA, 0.05 M sodium cacodylate buffer and 2% PFA used overnight, was the best approach to fix Ulvacean seaweeds for TEM. This approach ensured that key membranes, such as the thylakoid membranes of chloroplast, remained intact. The optimum post-fixation time was 8 h in 1% OT and 0.05 M sodium cacodylate buffer. I strongly suggest the use of ethanol in the dehydration process for Ulvacean seaweeds. This new protocol will ensure the ultrastructure architecture of Ulvacean seaweeds can be clearly ascertained using for transmission electron microscope, TEM, analysis.

Table 4.1. Global references of methods applied for Transmission Electron Microscopy (TEM) in diverse species of seaweeds.

Reference	Stage	Species	Fixation solution	Time	Post-fixation	Time	Dehydration
Bouzon et al. (2011)	Female plants	*Gracilariopsis tenuifrons*	2.5% Glutaraldehyde + 0.1 M Cacodylate phosphate (pH 7.2) + 0.2 M Sucrose	Overnight	1% Osmium tetroxide	4h	Acetone
Costa et al. (2016)	Thallus	*Sargassum cymosum*	2.5% Glutaraldehyde + 0.1 M Cacodylate phosphate (pH 7.2) + 0.2 M Sucrose	Overnight	1% Osmium tetroxide	4h	Acetone
Gasulla et al. (2013)	-	*Asterochloris erici*	2% Glutaraldehyde + Formaldehyde	4 h	1% Osmium tetroxide	2h	Ethanol
Holzinger et al. (2006)	Adult	*Prasiola crispa*	2% Glutaraldehyde + 0.05 M Cacodylate (pH 7)	2 h	1% Osmium tetroxide + Cacodylate buffer	4h	Ethanol
Holzinger et al. (2009)	Epipelic mats	Zygnema sp.	2% Glutaraldehyde + 0.01M Cacodylate (pH 6.8)	1 h	1% Osmium tetroxide +0.01M Cacodylate buffer	12h	Ethanol
Holzinger et al. (2010)	-	*Zygogonium ericetorum*	1.25% Glutaraldehyde + 0.01M Cacodylate (pH 6.8)	1.5 h	1% Osmium tetroxide + 0.01M Cacodylate	14-17 h	Ethanol
			2.5% Glutaraldehyde + 0.05 M Cacodylate	1.5 h	1% Osmium tetroxide + 0.05 M Cacodylate	14-17 h	Ethanol
Holzinger et al. (2011)	Algal filament	*Klebsormidium crenulatum*	1.25% Glutaraldehyde + 0.05 M Cacodylate (pH 6.8)	1 h	1% Osmium tetroxide + 0.01M Cacodylate	14h	Ethanol
Holzinger et al. (2011a)	Fertil sporophyte	*Saccharina latissima*	2.5% Glutaraldehyde + 0.01M Cacodylate (pH 6.5) + caffeine + 0.1% $CaCl_2$	2 h	1% Osmium tetroxide + 0.01M Cacodylate	16 h	Ethanol
Holzinger et al. (2015)	Thallus	*Ulva compressa*	2.5% Glutaraldehyde + 0.05 M Cacodylate (pH 6.8)	1.5 h	1 % Osmium tetroxide	18 h	Ethanol
Karsten and Holzinger (2012)	-	*Klebsormidium dissectum*	1.25% Glutaraldehyde + 0.05 M Cacodylate (pH 6.8)	1 h	1% Osmium tetroxide + 0.01M Cacodylate	14h	Ethanol
Løvlie and	Sporophytes	*Ulva mutabilis*	2% Glutaraldehyde + 2% Formaldehyde + 0.1 M Cacodylate buffer (pH 7.5)	6.5 h	2% Osmium tetroxide + 0.1 M buffer + NaCl	13 h	Ethanol

Brånten (1970)							
Maier (1997)	Male gametophyte	*Ectocarpus siliculosus*	2.5% Glutaraldehyde + 0.08 M Cacodylate (pH 7.4) + 0.4% caffeine + 70% Growing medium	80 min	1% Osmium tetroxide + 75% buffer	1 h	Acetone
Mogi et al. (2008)	Sporophytes	*Ulva compressa*	3% Glutaraldehyde + 0.1 M Cacodylate (pH 7.2)+ 2% NaCl + 0.1% $CaCl_2$	Overnight	2% Osmium tetroxide + 0.1M Cacodylate	2 h	Ethanol
Murakami and Packer (1970)	Thallus	*Ulva sp.*	2.5% Glutaraldehyde + 0.05 M phosphate (pH 7.4)	1 h	1% Osmium tetroxide + 0.05 M phosphate buffer	1 h	Ethanol
	Thallus	*Porphyra sp.*	2.5% Glutaraldehyde + 0.05 M phosphate (pH 7.4)	1 h	1% Osmium tetroxide + 0.05 M phosphate buffer	1 h	Ethanol
Pellegrini (1980)	Adult	*Cystoseira stricta*	5-6% Glutaraldehyde + 0.05 - 0.1 M phosphate (pH 7.3)	8 h	2% Osmium tetroxide + 0.05-1 M phosphate buffer	7 h	Ethanol/ Acetone
Polo et al. (2014)	Thallus	*Sargassum cymosum*	2.5% Glutaraldehyde + 2% Paraformaldehyde + 0.075 M Cacodylate phosphate (pH 7.2) + 0.005 M $CaCl_2$ + 0.2 M sucrose + 1% caffeine	Overnight	1% Osmium tetroxide	4 h	Acetone
Roleda et al. (2010)	Thallus	*Urospora penilliciformis*	2.5% Glutaraldehyde + 0.05 M phosphate (pH 7.2) + filtered seawater	2 h	1% Osmium tetroxide + 0.05 M Cacodylate	Overnight	Ethanol
Rover et al. (2015)	Zygotes and embryos	*Sargassum cymosum*	2.5% Glutaraldehyde + 2% Paraformaldehyde + 0.075 M Cacodylate phosphate (pH 7.2) + 0.005 M $CaCl_2$	8 - 12 h	2% Osmium tetroxide + 0.075 M phosphate buffer + 0.005 M $CaCl_2$	4 h	Acetone
Simioni et al. (2014)	Young gametophytes	*Gelidium floridanum*	2.5% Glutaraldehyde + 0.1 M Cacodylate phosphate (pH 7.2) + 0.2 M Sucrose	Overnight	1% Osmium tetroxide	4h	Acetone
Schiavon et al. (2012)	Thallus	*Ulva sp.*	3% Glutaraldehyde + 0.1 M Cacodylate (pH 6.9)	Overnight	1% Osmium tetroxide + Cacodylate buffer 0.1M	2h	Ethanol
Schmidt et al. (2009b)	-	Kappaphicus alvarezii	2.5% Glutaraldehyde + 0.1 M Cacodylate phosphate (pH 7.2) + 0.2 M Sucrose	Overnight	1% Osmium tetroxide	4h	Acetone
Steinhoff et al. (2008)	Young gametophytes	*Laminaria hyperborea*	2.5% Glutaraldehyde + 0.05 M phosphate (pH 7.2) + filtered seawater	4 h	1% Osmium tetroxide + 0.1M Cacodylate	4h	Ethanol

Table 4.2. Methods evaluated in *Ulva lactuca* for Transmission Electron Microscopy (TEM).

	Method A		**Method B**		**Method C**		**Method D**		**Method E**	
Stage	**Reagents**	**Time**	**Reagents**	**Time**	**Reagents**	**Time**	**Reagents**	**Time**	**Reagents**	**Time**
Fixation	2.5 % Glutaraldehyde 0.1 M Cacodylate 0.2 M Sucrose	Overnight	2.5 % Glutaraldehyde 0.1 M Cacodylate	4 h	2.5 % Glutaraldehyde 0.1 M Cacodylate	Overnight	2.5 % Glutaraldehyde 0.05 M Cacodylate 2% Paraformaldehyde	4 h	2.5 % Glutaraldehyde 0.05 M Cacodylate 2% Paraformaldehyde	Overnight
Post-Fixation	1% Osmium tetroxide 0.1 M Cacodylate buffer	4 h	1% Osmium tetroxide 0.1M Cacodylate buffer	Overnight	1% Osmium tetroxide 0.1M Cacodylate buffer	8 h	1% Osmium tetroxide 0.05 M Cacodylate buffer	Overnight	1% Osmium tetroxide 0.05 M Cacodylate buffer	8 h
Dehydration	Acetone	30 min	Ethanol	30 min	Ethanol	30 min	Ethanol	30 min	Ethanol	30 min
Infiltration	Acetone: Spurr's	24 h	Ethanol: Spurr's	24 h	Ethanol: Spurr's	24 h	Ethanol: Spurr's	24 h	Ethanol: Spurr's	24 h

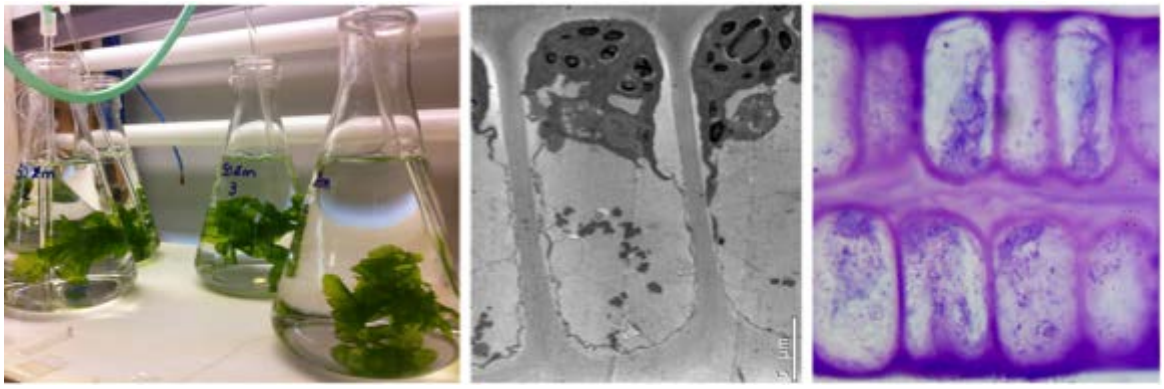
Table 4.3. Comparative table of the cell components affected by different fixation method evaluated in *Ulva lactuca*.

Method/ Cell component	Method A	Method B	Method C	Method D	Method E
Cellulose microfibrils	+	++	++		+++
Cell membrane collapse	+				
Lipid bodies		++		+++	+++
Mitochondrion				++	
Pyrenoid	+	++			+++
Starch granules	+		+	+	
Thilakoids membrane	+	+	+	+	+++
Vacuolo					

+ poor preservation; ++ preserved; +++ well preserved

CHAPTER 5

Photosynthetic and ultrastructural responses of *Ulva australis* to Zn stress



Preface:

The goal of this investigation was evaluated under laboratory conditions physiological, photosynthetic and ultrastructural responses of *U. australis* to zinc (Zn) pollution, where three Zn concentrations were assessed. The aim was to evaluate the potential of this species for biomonitoring and bioremediation from a physiological perspective because understanding metal resistance/ tolerance will be the base to determine the potential of *Ulva* for future applications.

It was identified that *U. australis* is a Zn-tolerant species, as metal stressors did not affect their physiology.

This work has been written according to a refereed scientific journal standard, and adapted for thesis requirements.

5.1 ABSTRACT

This research evaluated the effect of zinc (Zn) on the ultrastructure and the photosynthetic efficiency of the common green alga *Ulva australis*. *U. australis* was grown in the laboratory for 7 days under a range of different Zn concentrations (0, 25, 50 and 100 $\mu g \cdot L^{-1}$). Growth rate (Gr), photosynthetic efficiency (Fv/Fm and ETR_{max}), photosynthetic pigments, and metal accumulation were measured. Samples of 1 mm length were taken to analyse the effect of Zn on ultrastructure using transmission electron microscopy (TEM) and cytochemical responses (TB-O, PAS and CBB) were evaluated by Light Microscopy (LM). There were no significant differences in the growth rate, Fv/Fm, ETR_{max}, metal accumulation, and the photosynthetic pigments chlorophyll *a*, chlorophyll *b* and carotenoids ($p > 0.05$) after 7 days of Zn exposure. However, TEM revealed cellular damage such as cytoplasm retraction, compression of cellulose fibrils, dissembled thylakoids and electron-dense bodies suggesting ultrastructural impacts from metal exposure and accumulation. The cellular damage increased when *U. australis* was exposed to higher Zn concentration. Cytological analysis demonstrated that Zn affected the normal performance of *U. australis* cells at the three concentrations tested. The main damage was cytoplasm retraction and a decrease in the amount of starch granules, following exposure at 25 $\mu g \cdot L^{-1}$ and 50 $\mu g \cdot L^{-1}$ of Zn. I conclude that concentrations of Zn assessed in *U. australis* in this research caused short-term cellular damage as revealed by TEM and cytological analysis, demonstrating the importance of measuring a broad suite of endpoints to better understand species responses to environmentally relevant concentrations of Zn. However, *U. australis* was able to physiologically acclimate to

adverse conditions, since there was no effect on the photosynthetic performance and growth.

Key words

Cytochemistry, cytology, metal pollution, photosynthesis, seaweeds.

5.2 INTRODUCTION

Various organisms have been studied, including benthic algae, to detect levels of metals contamination in coastal systems. Natural populations of seaweeds have been shown to be valuable indicators of local metal contamination, and their use as biomonitoring tools has been well documented (Boubonari et al. 2008; Stengel et al. 2005; Zbikowski et al. 2007; Brown et al. 1999; O'Leary and Breen 1997; Whitton and Kelly 1995; Malea et al. 1994; Malea 1994; Ho 1990; Al-Homaidan et al. 2011). Green seaweeds, such as *Ulva* spp, are popular indicators of pollution and common bioindicators worldwide. However, a bioindicator depends on both the local stressors, and the species representative of a particular environment (Zhou et al. 2008), thus making every bioindicator species specific for a particular environment.

Metal concentrations in seaweeds can vary depending on a range of physiological factors such as the age of the algae thalli (old or new tissue), and the external (thallus, blade or stipe) and/or internal cellular organisation. Investigation of algal physiology is important to better understand whether the internal architecture might be influenced by metal accumulation. Previous studies on brown seaweeds suggest that metal concentration might affect physiological conditions and photosynthetic efficiency (Connan and Stengel 2011). More recent investigations demonstrated that lead (Pb) and copper (Cu) negatively affects morphological, biochemical and physiological processes in the red algae *Gracillaria domingensis* (Gouveia et al. 2013) and in *Gelidium floridanum* (Santos et al. 2014). Although there has been some research on green seaweeds (Schiavon et al. 2012; Vecchia et al. 2012), still little is known about metal effects on their physiology. Consequently, it is important to assess if metals affect the physiology and ultrastucture of algae that are to be used in biomonitoring programs.

The effects of heavy metals on the physiology and ultrastructure of different seaweed species have been assessed *in vitro*. For instance, the photosynthetic performance and relative electron transport rate (rETR) of the red seaweed *Hypnea musciformis* decreases when plants are exposed to cadmium (Cd), and the cellular ultrastructure is also adversely affected (Bouzon et al. 2011). A more recent study demonstrated that copper (Cu) and lead (Pb) also affect the electron transport rate (ETR) of the brown seaweed *Sargassum cymosum* when exposed to these metals (Costa et al. 2015). Similarly, Santos et al. (2012) demonstrated that pigment content changed in *G. domingensis* when exposed to Cd, Cu, and Pb, and that exposure to these metals produces an adverse effect on organelles (i.e. chloroplasts). These findings highlight the importance of microscopy as an analytical tool in physiological studies to demonstrate the changes that can occur at a cellular and organelle level and how these might affect physiological responses.

Zinc (Zn) is an essential micronutrient for all living organisms, and is a vital for cell functions and components of enzymes and proteins (Hall 2002). Zinc is a co-factor for the ubiquitous enzyme carbonic anhydrase (Raven et al. 1999), which is crucial for regulation of photosynthesis (Tsuzuki and Miyachi 1989). However, in high concentrations Zn can be lethal for some organism (Hall 2002).

Zinc can enter the waterways via industrial discharge, urban or aquaculture effluents. The Derwent Estuary in Tasmania, Australia, is strongly affected by industrial discharge and it has high levels of heavy metals in sediments, water and biota (Coughanowr et al. 2015). In fact, this estuary has been declared as the most polluted estuary in Australia (Wood et al. 1992; Bloom and Ayling 1977; Whitehead et al. 2010). However, high concentrations of metals in sediment and seawater of the Derwent have decreased since the first data

were recorded in 1972 (Whitehead et al. 2010; Wood et al. 1992; Bloom and Ayling 1977; Coughanowr et al. 2015). Zinc is the most prevalent metal contaminant in the Derwent Estuary (Coughanowr et al. 2015), and can reach 50 $\mu g \cdot L^{-1}$ in seawater, and 14 600 $mg \cdot kg^{-1}$ in sediments. Recent monitoring of macroalgal species in the Derwent showed elevated metal content in *Ruppia megacarpa* (635 $mg \cdot kg^{-1}$ Dried Matter Basis (DMB)) and in *Ulva australis* (321 $mg \cdot kg^{-1}$ DMB).

Although the effects of metals in seaweeds have been well investigated worldwide, little is known about Zn toxicity in seaweed (Hurd et al. 2014). The physiological response of *Ulva* to Zn is unknown and therefore, I aim to evaluate *in vitro* both the physiological and ultrastructural responses of *U. australis* to Zn as a chemical stressor.

5.3 METHODS

- **Sampling and experiment preparation**

All glassware and plasticware used for preparation and experimentation with metal solution were washed with alkaline detergent Decon 90® 2 % solution, and acid bath 5 % HNO_3 for at least 24 h. All material was then rinsed with Milli-Q® water (18 MΩ) and dried in an oven.

Ulva australis was collected in June 2014 from Adventure Bay, Bruny Island, Tasmania, Australia (43° 21' 53" S; 147° 21' 3" E) - an intertidal unpolluted area. Approximately 300 g of fresh seaweed was transported to the laboratory in an insulated container. Samples were then kept in a 20 L container with seawater and aeration (14 °C ± 1 °C) for one week for prior to the experiment set-up.

- **Experimental setup**

Approximately three grams (2.84 ± 0.1 g) of plant material (individual thalli) were placed in a 2 L plastic jar at 14 °C ± 1 °C with 1 µm filtered seawater (SW) 33 ‰ and constant aeration. Light was provided at 100 $\mu mol^{-1} \cdot m^{-2} \cdot sec^{-1}$ with a photoperiod of 12L: 12D. Individual jars were exposed at 3 graded concentrations spiked with a $ZnCl_2$ stock solution of 1 $mg \cdot L^{-1}$; 0 $\mu g \cdot L^{-1}$ cControl (C), 25 $\mu g \cdot L^{-1}$, 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$, for seven days, with 5 replicates for each treatment and control (n = 5). Experimental Zn concentrations were selected to reflect Zn concentrations observed in surface seawater in the Derwent Estuary, (25 $\mu g \cdot L^{-1}$ and 50 $\mu g \cdot L^{-1}$) with a higher concentration (100 $\mu g \cdot L^{-1}$) also included as a higher-level reference. Von Stoch medium (No EDTA) was added to the control and treatments to provide the nutrients necessary for growth (Nitrate ($NaNO_3$) and phosphate (Na_2HPO_4) at 250 µM and 7.105 µM concentration, respectively). There was only nominal Zn concentrations in the control seawater.

- **Growth rate (Gr)**

Wet weight (WW) was taken at the start and end of the experiment in order to obtain the growth rate (GR) over the experimental period, which was calculated according to Yong et al. (2013). Any change in coloration or visual damage was recorded and considered as a result of the treatments.

- **Photosynthetic performance**

Fluorescence was measured using a Pulse-Amplitude Modulate (PAM) Diving fluorometer (Walz, Effeltrich, Germany). A pilot trial was run to determine the appropriate configuration of the PAM parameter (Gain = 4, Light intensity = 2 and

Measuring light intensity = 4). Samples were acclimated in the dark-adaptor clip for 20 min prior to measurements.

To obtain the maximum quantum yield (Fv/Fm) of photosystem II (PSII) the following parameters were measured: maximum fluorescence for dark-adapted tissue (Fm), the minimum fluorescence for dark-adapted tissue (Fo), and the maximum fluorescence (Fv). The maximal relative rate of electron transport activity (ETR_{max}, $\mu mol \cdot electrons \cdot m^{-2} \cdot s^{-1}$) (Longstaff et al. 2002), was calculated from the rapid light curves (RLC) described in Ralph and Gademann (2005) using the exponential curve model of Platt et al. (1980). The parameters were calculated by the PAM processor version 1.0 (PAM-Processor 2015) using R Core Team (2013)

- **Photosynthetic pigments**

Chlorophyll *a* and *b*, and carotenoids were analysed for each treatment. Pigments were extracted from ~0.5 g of *U. australis* biomass (n = 5) in 3 ml dimethylsulfoxide 99% (DMSO) for 1 h at 40 °C in an oven, following Schmidt et al. (2010). 200 µL of supernatant was added into a 96 wells micro-plate (IWAKI). A micro-plate reader (Biotek synergy^{TM} HT) was used to determine the absorbance of different components such as chlorophyll *a* (Chl *a*: 663 nm), chlorophyll *b* (Chl *b*: 645 nm) and carotenoids (car: 480 nm). Pigments were quantified according to Wellburn (1994), and results for all photosynthetic pigments are expressed in $\mu g\ g^{-1}$.

- **Ultrastructure**

Samples of 1 mm length were taken at the end of the experiment from each treatment (*n* = 5) for microscopic analysis. Samples were placed in a vial with 2 mL 2.5 % glutaraldehyde, 0.05 M sodium cacodylate buffer (pH 7.2), and 2 % paraformaldehyde for overnight fixation for transmission electronic microscopy (TEM). Samples were post-fixed with 1 % osmium tetroxide and 0.05 M sodium cacodylate for 8 h, followed by dehydration in an ascending ethanol series (See Chapter 3). Finally, samples were embedded in Spurr's resin (Low viscosity, ProScitech) to produce a solid block, which was cut with an ultramicrotome (Leica EM UC7) at 60 nm for the transmission electron microscope (JEOL JEM-1011 TEM). Block cutting and TEM analysis were performed at the Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, Brazil.

- **Cytochemistry**

Cytochemistry is an approach for identifying subcellular compounds such as proteins and carbohydrates using chemical stains (Gahan 1984). For this approach 1 mm thick samples were fixed in 2 ml of 2.5 % paraformaldehyde and 0.1 M (pH 7.2) phosphate buffer following the methods described by Schmidt et al. (2009a). Samples were dehydrated using an ascendant ethanol succession and embedded in historesin (Leica Historesin, Heidelberg, Germany) to create a block. Historesin blocks were sectioned at 5 μm with a microtome to produce a slide with transverse sections of *Ulva* tissue (RM 2135 Leica Leitz). Slides were stained with Toluidine blue (TB-O) to identify acid polysaccharides, Periodic Acid-Schiff (PAS) for identifying basic polysaccharides (starch and cellulose), and Coomassie Brilliant Blue (CBB) for observing cellular structure with a Light Microscopy (LM) with an Epifluorescent microscope Olympus BX 41, equipped with

Image Q Capture Pro 5.1 software (Qimaging Corporation, Austin, TX, USA). Slide cutting, stain, and LM analysis were completed at the Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, Brasil.

- **Metal concentration**

Ulva thalli were collected from an unpolluted area. Before the laboratory experiment began, total Zn levels were tested and metal content in seaweed (8.0 ± 0.1 $mg \cdot kg^{-1}$ DMB), seawater (4.30 ± 1.58 $\mu g \cdot L^{-1}$) and sediments (17.25 ± 0.5 $mg \cdot kg^{-1}$ DMB), were low and indicative of unpolluted environments. Samples of *U. australis* were kept in a freezer at -24 °C until dried in a freeze-drier (FreeZone 4.5 Labconco) for 24 h, until a constant dry weight (DW) was obtained. Samples were ground with a coffee grinder to obtain < 2 mm particles for analysis. For metal analysis, dry samples were sent to Analytical Services Tasmania (AST) a NATA (National Association of Testing Authorities, Australia) certified laboratory. Zinc content in thalli was detected using an Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Metal detection limit for Zn was 0.1 $mg \cdot kg^{-1}$ WMB (wet matter biomass). Laboratory certified standard reference material (CRM) was used to test the accuracy and precision of the analysis.

- **Statistical analysis**

A one-way ANOVA was conducted (95% confidence interval) to evaluate differences in physiological parameters, growth rate, metal concentration, photosynthesis performance and photosynthetic pigments, between treatments. Statistical analysis was performed using SPSS IBM Statistic version 22 (IBM 2013).

5.5 RESULTS

- **Growth rate (Gr)**

There was no significant difference between the growth rate of the control algae and any treatments (Table 5.1) ($p > 0.05$, $F = 0.545$, $df = 3$) after 7 days of Zn exposure. Similarly, there was no evidence of damage to the thalli under any treatment. However, there was a positive growth rate in control thalli, but some treated thalli showed negative growth rates.

Table 5.1. Growth rate (% day^{-1}) and metal concentration ($mg \cdot kg^{-1}$ DMB) of *Ulva australis* exposed to different Zn concentrations. Values mean ± SE.

Treatment/ Parameter	Growth rate (% day^{-1})	Zn ($mg \cdot kg^{-1}$)
Control	1.724 ± 0.81	8 ± 0.01
25 $\mu g \cdot L^{-1}$	0.645 ± 1.03	8.9 ± 0.01
50 $\mu g \cdot L^{-1}$	0.369 ±1.36	8.4 ± 0.01
100 $\mu g \cdot L^{-1}$	0.096 ± 0.34	8.7 ± 0.01

- **Photosynthesis performance**

The increasing Zn concentration did not have any effect on photosynthesis with no significant differences in the two fluorescent parameters evaluated (Table 5.2), maximal photochemical yield (Fv/Fm), and maximum electron transport rate (ETR_{max}) ($p > 0.05$, $F = 0.911$, $df = 3$).

- **Photosynthetic pigments**

Similarly, photosynthetic pigments, Chl *a*, Chl *b*, total *Chl* and carotenoids, were also not significantly affected by different Zn concentrations after 7 days of exposure (Table 5.2) (ANOVA $p > 0.05$, $F = 1.05$, $df = 3$).

Table 5.2. Photosynthetic parameters (Fv/Fm and ETR_{max} ($\mu mol \cdot electron \cdot m^{-2} \cdot s^{-1}$)) and photosynthetic pigments (Chl *a*, Chl *b* and carotenoids, ($\mu g \cdot g^{-1}$) of *Ulva australis* exposed at different zinc (Zn) concentrations (Control, 25 $\mu g \cdot L^{-1}$, 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$) after 7 days of exposure. Values are expressed as mean ±SE ($n = 5$).

Treatment/ Parameter	**Fv/Fm**	**ETR_{max} ($\mu mol \cdot electron \cdot m^{-2} \cdot s^{-1}$)**	**Chl *a* ($\mu g \cdot g^{-1}$)**	**Chl *b* ($\mu g \cdot g^{-1}$)**	**car ($\mu g \cdot g^{-1}$)**
Control	0.775 ± 0.01	16.28 ± 1.3	48.74 ± 12.4	337.51 ± 84.8	7.58 ± 1.4
25 $\mu g \cdot L^{-1}$	0.758 ± 0.02	12.54 ± 1.2	85.78 ± 27.3	369.05 ± 65.6	12.08 ± 2.6
50 $\mu g \cdot L^{-1}$	0.776 ± 0.01	14.99 ± 1.3	45.39 ± 24.3	214.86 ± 76.6	6.67 ± 2.9
100 $\mu g \cdot L^{-1}$	0.773 ± 0.01	18.14 ± 0.7	51.09 ± 10.6	307.11 ± 52.5	8.35 ± 1.7

- **Ultrastructure**

There were however, ultrastructre differences following Zn exposure, with the cell ultrastructure differing markedly between the control and treated thalli (Fig. 5.1 and 5.2). Cell components such as chloroplasts, mitochondria, starch grains, lipids bodies, Golgi, nuclei, and a large vacuole were well organised and visible in the control plants (Fig. 5.1a). High-magnification of the cell wall clearly shows the parallel organisation of the cellulose fibrils in the control plants (Fig. 5.1b) and material deposited in the Golgi bodies (Fig. 5.1c) was evident in the higher resolution image of this organelle (Fig. 5.1d). Mitochondria were well organized in the control cell (Fig. 5.1e), and the parallel thylakoids membranes of the chloroplast were well preserved (Fig. 5.1f-g). A starch grain surrounded by thylakoids was also observed (Fig. 5.1f).

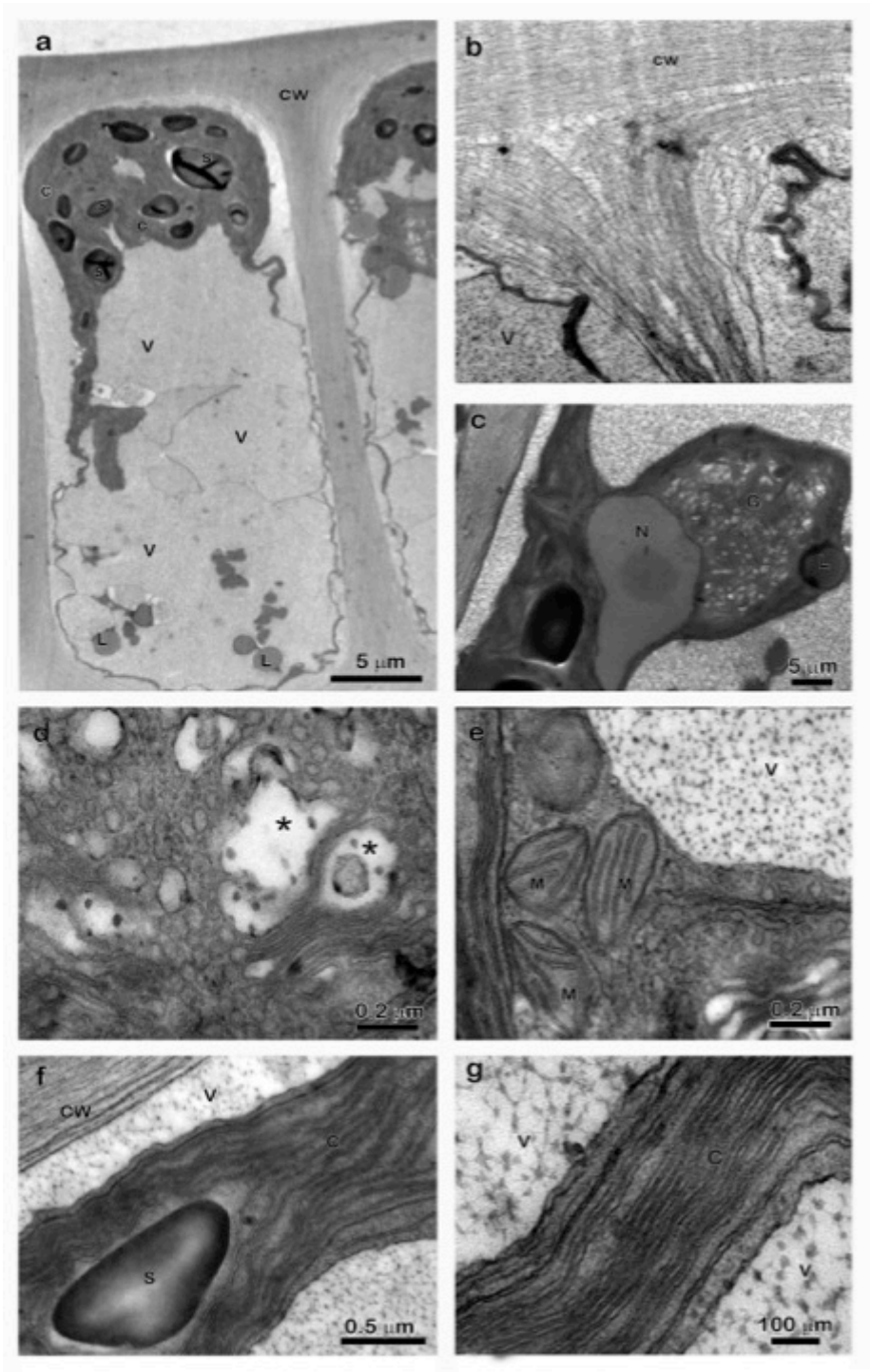


Figure 5.1. Ultrastructure analysis by electron microscopy of control plants of *Ulva australis*; a) cell with internal components well organised, b) cell wall, external layer and internal division of two cells, c) Golgi next to nucleus, d) amplification of Golgi material indicated by *, e) mitochondria in the chloroplast, f) chloroplast surrounding a starch granule, g) thylakoids fibrils of chloroplast organisation. C: chloroplast, CW: cell wall, G: Golgi, L: lipids, M: mitochondrion, N: Nucleus, P: pyrenoid, S: starch granules, V: vacuole.

After Zn exposure, the treated cells showed cytoplasm retraction and visible compression of the cellulose fibrils of the cell wall (Fig. 5.2b, e and i) in relation to the control. Electron-dense bodies were observed in the cell wall and vacuole (Fig. 5.2g, j, k). Deteriorating thylakoids were detected in all treated thalli (Fig. 5.2c), and this effect clearly increased when Zn concentration increased (Fig. 5.2e and j). A few plastoglobuli were identified on the internal cavity of the vacuoles at 25 and 50 $\mu g \cdot L^{-1}$ of Zn concentration (Fig. 5.2e and f). In thalli exposed to 100 $\mu g \cdot L^{-1}$ of Zn, the number of mitochondria increased in relation to the control thalli (Fig. 5.2i).

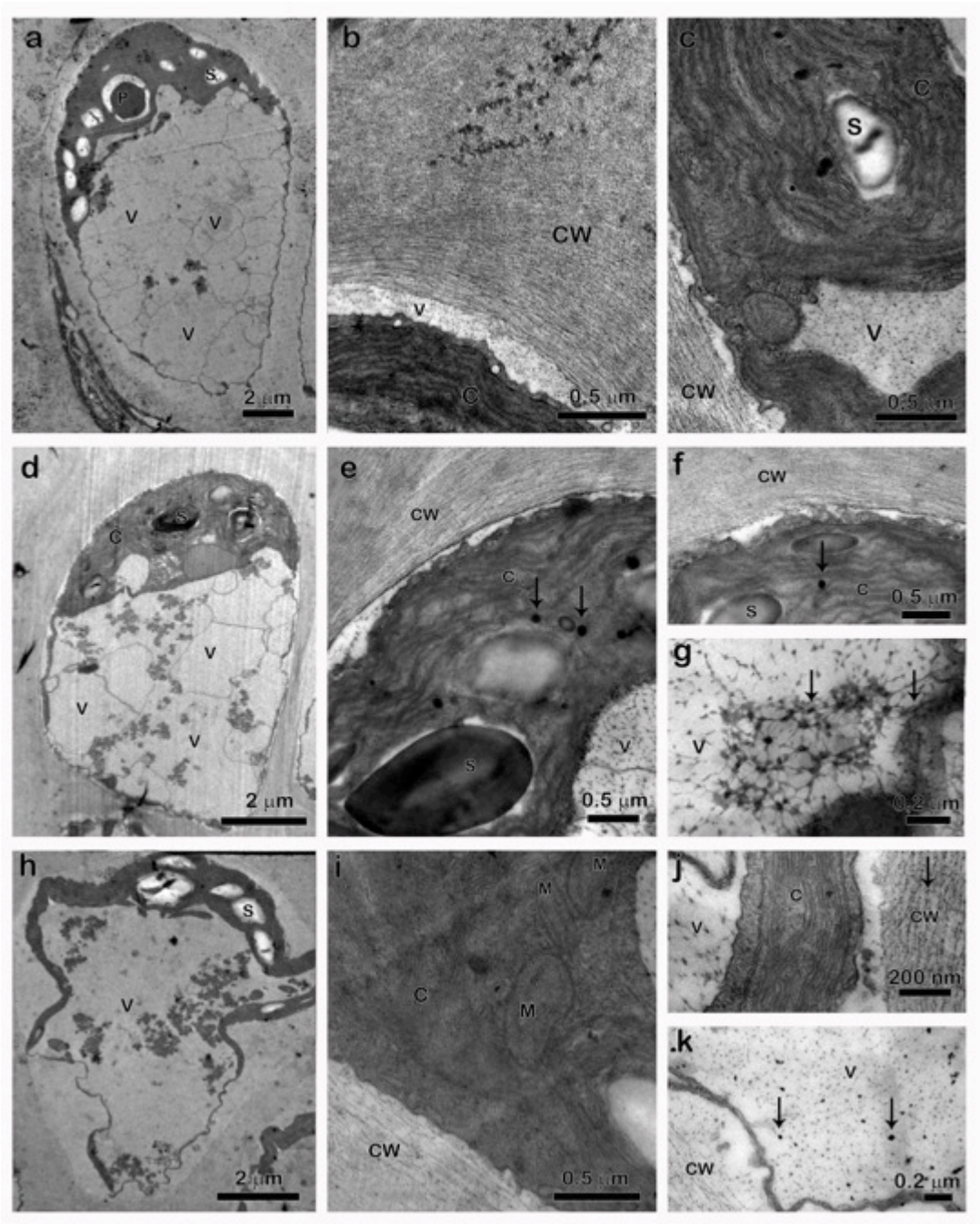


Figure 5.2. Ultrastructure analysis by transmission electron microscopy of exposed plants of *Ulva australis*; a-c) details of cell affected by 25 µg·L^{-1} Zn showing internal components organisation and cellular shape slightly affected by metal, b) compression of cellulose fibrils, and c) normal chloroplast. d-g) details of cell and organelles treated by 50 µg·L^{-1} Zn showing an alteration on cell shape, e-f) plastoglobulli on the chloroplast and vacuole (arrows), g) electron-dense material (arrows). h-k) details of organelles treated by 100 µg·L^{-1} Zn, h) clear cytoplasm retraction, i) increase number of mitochondria, and j-k) electron-dense bodies indicated by arrows on vacuole and cell wall. C: chloroplast, CW: cell wall, G: Golgi, L: lipids, M: mitochondrion, P: pyrenoid, S: starch granules, V: vacuole.

- **Cytochemistry**

Cross sections of the control thalli of *U. australis* showed two parallel cell layers, typical of organisation within Ulvacean (Kraft et al. 2010). A positive metachromatic reaction was visible in tissue stained with Toluidine blue (Fig. 5.3a). Periodic Acid-Schiff (PAS) stained cells also showed a strong positive reaction and clearly demonstrated the cellulosic compounds in the cell wall, and the presence of marginal starch grains, and neutral polysaccharides, which are a reserve substance (Fig. 5.3b). Vegetal tissues stained with Coomassie Brilliant Blue showed a normal cell configuration and a positive reaction of proteins indicated by the lipids drops (Fig. 5.3c).

Cells of treated thalli indicated a positive reaction to PAS and showed the retraction of the cytoplasm (Fig. 5.3e, h and k). There was a decrease in the number of starch granules at both 25 $\mu g \cdot L^{-1}$ and 50 $\mu g \cdot L^{-1}$ (Fig. 5.3e and h). Marginal organisation of starch granules was visible (Fig. 5.3k) at the highest Zn concentration (100 $\mu g \cdot L^{-1}$). In the treatment, cell alteration was visible when samples were stained with CBB, and there was a positive stain reaction clearly showing that the distribution of the proteins components through the cell differs from control cells (Fig. 5.3f, i and l).

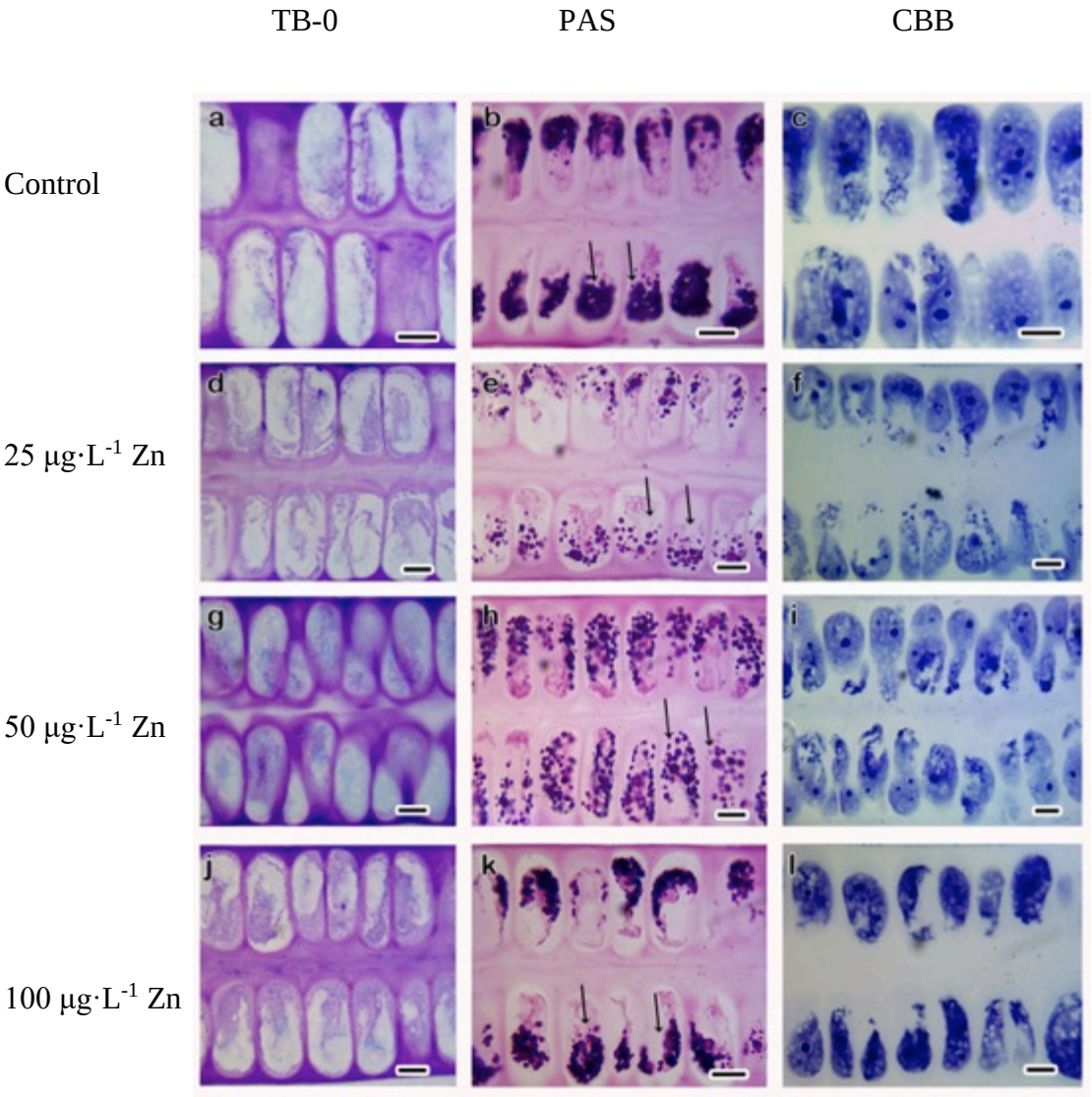


Figure 5.3. Light microscopy analysis of transversal-sections of *Ulva australis*; a, d, g, j: samples stained with Toluidine Blue (TB-O), indicating metachromatic reactions. b, e, h, k: samples stained with Periodic Acid-Schiff (PAS) indicating a positive reaction with starch grains, cell wall polysaccharide and cytoplasm retraction of treated cell. c,f,i,l: samples stained with Coomassie Brilliant Blue (CBB) showing positive reaction, with nucleus and proteins. Scale bar for all figures 10 µm.

- **Metal concentration in seaweed**

Metal content in the seaweed did not differ ($p > 0.05$) between treatments when exposed to the different metal concentrations (Table 5.1).

5.5 DISCUSSION

This study showed that there were clear ultrastructural and cytological changes in *U. australis* when exposed to environmentally relevant Zn concentrations. These changes include chloroplast alteration, presence of electron-dense bodies, cytoplasm retraction and decreased numbers of starch granules. However, despite these marked changes to the cellular structure, there were no significant effects of Zn exposure on growth, pigment content, maximal photochemical yield (Fv/Fm), maximum electron transport rate (ETR_{max}) or metal uptake. These findings suggest *U. australis* can cope with relatively high levels of damage to cell structure, without affecting growth or photosynthetic performance. This lack of physiological responses to clear cell damage, has been observed in other species of Ulvaceans. For instance, a previous study showed that there was no change in the gross photosynthesis rate for *U. flexuosa* (formerly *Enteromorpha flexuosa*) when exposed to 50 $\mu g \cdot L^{-1}$ of Cu, but that electron-dense deposits and an increase in vacuoles in the cytoplasm associated with Golgi was apparent after 5 days of metal exposure (Andrade et al. 2004). Other species such as *U. intestinalis* accumulate electron-dense bodies in the cytoplasm induced by Cu contamination (Correa et al. 1999), *U. intestinalis* and *U. laetevirens* also show cellular damaged when exposed to different Cd concentrations (Vecchia et al. 2012). *Ulva intestinalis* is more susceptible, with clear damage to the thylakoid systems of the chloroplast, while *U. laetevirens* show irregularities in cell shape (Vecchia et al. 2012).

In contrast to our study, other species of Ulvaceans such as *U. lactuca* and *U. fluxuosa*, have reduced growth rates as a result of exposure to two different Zn concentrations (100 $\mu g \cdot L^{-1}$ for 7 days and 20 $\mu g \cdot L^{-1}$ for 15 days) (Amado Filho et al. 1997). Studies have shown that other metals, such as Cu, can also affect the normal growth rate of *U. pertusa*

at relatively low Cu concentrations (25 μg·L^{-1}), while in *U. armonica* the growth is only affected at high concentrations of Cu 100 μg·L^{-1} (Han et al. 2008). *Ulva prolifera* and *U. linza* have negative growth rates when exposed to Cd over 4.5 μg·L^{-1} (Jiang et al. 2013). Therefore, this variability on the metals content in different *Ulva* species, demonstrated the adaptability of every organism and their distinctive respond to metal pollution, even though they are very close related.

In this study, the photosynthetic performance and photosynthetic pigments of *U. australis* were not affected when thalli were exposed to different Zn concentrations under controlled conditions. Baumann et al. (2009) observed that in *U. intestinalis* and other seaweed species after four days of exposure (Zn at 6.5 μg·L^{-1}) reduce the maximum quantum yield (Fv/Fm), and confirmed thallus death by the Fv/Fm = 0. Han et al. (2008) discovered differences in the photosynthetic activity of *U. armonica* and *U. pertusa* exposed to high concentrations of Cu (250 μg·L^{-1}), but there were no differences at concentrations <100 μg·L^{-1} of Cu. Similar results were described for *E. fluxtuosa*, where the photosynthetic process was also disturbed at concentrations higher than 250 μg·L^{-1} of Cu (Andrade et al. 2004).

In general, I observed that there was a slight increase in Chl *a* and carotenoids, in conjunction with a slight reduction of Fv/Fm and ETR_{max} after 7 days of Zn exposure at 25 μg·L^{-1}. In essence, it seems that the plant is responding to the new environmental conditions and adapting. Metals can induce the production of carotenoids (Pinto et al. 2003). Chl *a* and carotenoid play a key role in photosynthesis. Chlorophyll is an essential pigment that transfers the energy necessary for this process (Graham et al. 2009), whilst, carotenoids protect the light-harvesting complex, which transfers light energy to the Chl *a*

molecule, to protect against photochemical damage (Pinto et al. 2003). An increase in carotenoids contents has been demonstrated to act as a defence strategy to prevent toxic effects in red algae exposed to Pb (Gouveia et al. 2013), and as antioxidant mechanism against metals (Santos et al. 2014).

In contrast, at 50 $\mu g \cdot L^{-1}$ and 100 $\mu g \cdot L^{-1}$ of Zn, pigments were shown to slightly decrease alongside a slight increase in photosynthetic activity, which demonstrates tolerance to both concentrations over the 7 days of exposure. However, this response did not differ significant between treatments. There are a number of similar examples to this chlorophyll and carotenoids concentrations declined in *U. prolifera* and *U. linza* when Cd concentrations increased, but these differences were not significant in either species at concentrations of 1.1 $\mu g \cdot L^{-1}$ (Jiang et al. 2013). In *U. armonica* exposed at different concentrations of Cu, Chl *a* and *b* increased at 50-100 $\mu g \cdot L^{-1}$ but there were no decreases in Fv/Fm at 100 $\mu g \cdot L^{-1}$; the increment in pigments was related to a compensation on energetic resources for growth (Han et al. 2008).

There is little information about the ultrastructural damage on Ulvaceans exposed to metal stress. This investigation provides an improved understanding of the physiological responses of Ulvales to metal pollutants in metal impacted environments, demonstrating that Zn is disturbing the internal architecture of *U. australis*, and indicating that cytoplasm retraction, cell wall compressing, disorganisation of the thylakoids and electron-dense bodies on the cell wall, are evidence of metal accumulation.

I observed cell wall compression in treated thalli on the two lowest concentrations of Zn assessed. This could be because the polysaccharide content varies (as detected by PAS

stain). Polysaccharides are the main component of the cell wall and are responsible for metal binding and accumulation (Bouzon et al. 2012). The production of polysaccharides can be affected by growing conditions (Layahe and Robic 2004). This metal-polysaccharides affinity could be associated with the structural conformation, morphological shape conformation, cell wall compression, as well as cytoplasm retraction evident in the ultrastructure and confirmed with the PAS stain in treated thalli. Similar results were detected with *U. laetevirens* exposed to Cd, where this metal produces cytoplasm vesiculation, alteration of thylakoids and anomalies of cell morphology, which was related to bonds between the metals ions and the components of the cell wall (Vecchia et al. 2012). Andrade et al. (2004) observed alterations in the chloroplast, with electron-dense bodies deposited in the cytoplasm and into vacuoles, and an increase of vesicles associated the Golgi apparatus in *U. flexuosa* thalli exposed to Cu at 50 $\mu g \cdot L^{-1}$. Electron-dense bodies were also found on metal treated thalli of the red algae *Gelidium floridanum* (Santos et al. 2014) and *H. musciformis* (Bouzon et al. 2012), in both cases electron-dense bodies were related to metal deposits.

The major damage observed in this research was on the thylakoids of treated plants. This structures are where the light-dependent reactions associated with photosynthesis occurs, and is where Chl *a* is located (Graham et al. 2009). This light-dependent activity can be measured as Fv/Fm. In this research, Zn affected the thylakoids in all treated thalli, but did not affect Fv/Fm or chlorophyll contents in treated thalli. This could indicate that the stressor only produces short-term damage on the ultrastructure of *U. australis*. Longer exposure time or higher metal concentrations may produce adverse effects on photosynthesis. On the other hand, we also observed an increase on plastoglobuli, the structure that stores proteins and lipids that contribute to chloroplast functions, that has

been related to oxidative stress in higher plants (Austin et al. 2006). In the green microalga *Chlorella vulgaris* exposed to Zn, the increase of plastoglobulis were specifically related to metal binding and chelating, as a mechanism to contribute to metal tolerance (El-Naggar and Sheikh 2014).

I observed a decrease in the grains starch at lower concentrations, and whilst in *U. fluxuosa* exposed at 250 $\mu g \cdot L^{-1}$ of Cu there is a clear increase in the starch grains (Andrade et al. 2004). Similar results were found by Bouzon et al. (2012) and (Santos et al. 2014), who reported an increase in floridean starch (the main form of carbon storage in red algae) grains in the red seaweeds (*H. musciformis* and *G. floridanum*, respectively*)* exposed to metal under laboratory conditions. However, major cellular damage was detected in thalli exposed to only 25 $\mu g \cdot L^{-1}$ of Zn, suggesting that the algae might quickly absorb any metal available in the environment. Santos et al. (2014) explains that metals can easily enter the cell wall before affecting pigments and organelles. I expect that such a finding would affect metal accumulation in algal tissue. However, I did not find any significant differences in the Zn accumulated in *Ulva* tissue after 7 days exposure.

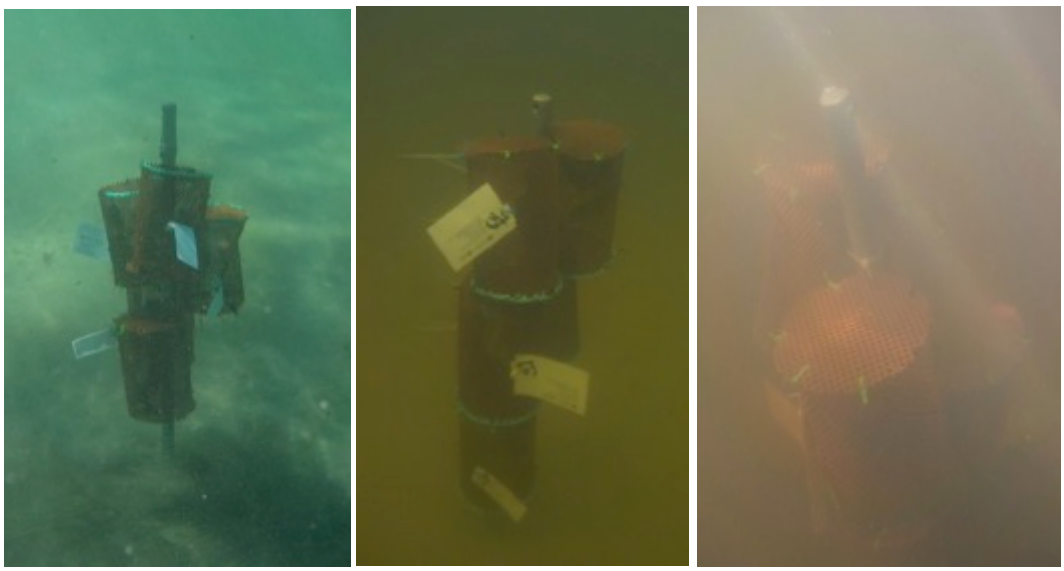
In general, it has been suggested that Fv/Fm is the most useful parameter to use for assessment of metal toxicity, rather than pigment contents (Chl *a*) and growth rate (Zakeri and Abu Bakar 2012). We recommended the analysis of internal components as a relevant parameter for metal toxicity, and that seaweeds can be a complementary addition to metal toxicity investigations.

5.6 CONCLUSIONS

Ulvacean algae are highly adaptable and tolerant, although every species is unique, and their tolerance to environmental stressors differs. My results show that *U. australis* can readily tolerate zinc concentrations in the short-term (7 days) under defined experimental conditions. There were no significant changes to growth rate, photosynthetic parameters, photosynthetic pigments or metal concentration after 7 days of metal exposure. However, differences in the internal cellular components of *U. australis* treated at different Zn concentrations, demonstrated visible alterations of the internal architecture of the treated thalli, and these is amplified when the Zn concentration increased. Therefore, the concentrations of Zn assessed in this research caused a short-term damage as evidenced by cytology studies, which clearly showed the importance of evaluating the internal architecture of seaweeds to better understand the effects of metal stressors.

CHAPTER 6

In situ assessment of *Ulva australis* as a monitoring and management tool for metal pollution



Preface:

This chapter addresses the application of a suitable bioindicator of pollution to be evaluated as a bioremediation tool for metal pollution. Currently, seaweeds are popular bioremediation tools for nutrient-enriched environments, but their potential for bioremediation of metal-polluted environments has been investigated. Small-scale *in situ* assessments proved the potential of a local species *Ulva australis*, to clean up a metal-polluted estuarine system.

This work has been published in the Journal of Applied Phycology (DOI: 10.1007/s10811-017-1073-y), special issue for the 22nd International Seaweed Symposium (ISS 2016). Therefore, this chapter is presented in similar publication form.

6.1 ABSTRACT

I investigated the extent of *Ulva australis* physiological responses to metals and their implications for biomonitoring and management tool. To determine the capacity of *Ulva* to accumulate metals over the short-term I undertook an *in situ* experiment where transplanted thalli to sites with different levels of metal pollution. After 12 days, arsenic, copper, lead and zinc accumulation was observed. Zinc was significantly greater ($p = 0.001$) at the most polluted site and was highly correlated ($r = 0.87$) with seawater total Zn concentration. Also assessed whether metal exposure can compromise *U. australis* performance by evaluating physiological responses and changes in thalli ultrastructure. We observed an increase in electro-dense bodies in the cell walls and vacuoles, which clearly indicate metal accumulation. However, there was no change in physiological performance (i.e. growth rate, Fv/Fm, ETR_{max}, photosynthetic pigments content) between the control and transplanted thalli ($p > 0.05$). Long-term bioremediation capacity of *U. australis* was assessed by deploying thalli at a highly polluted site for 45 days, where zinc in *Ulva* markedly increased over time, and was highly correlated with the seawater concentrations ($r = 0.85$). The metal uptake rate increased steadily over time, confirming that *Ulva* is clearly capable of bioaccumulation. However, visual examination of the thalli suggested degradation over time, which might limit deployment time (20 days). Clearly, *U. australis* has potential as a biomonitor tool, particularly for zinc, but the results suggest it may also be a useful tool for removing metals from the environment.

Key words

Bioaccumulation, photosynthetic performance, physiological performance, ultrastructure, zinc.

6.2 INTRODUCTION

Heavy metal pollution is a worldwide concern, with a broad range of anthropogenic activities contributing metals to our environment, resulting in long-lasting impacts on aquatic ecosystems (Bird et al. 1998). As the human population has increased exponentially, the impact of contaminants on the environment has also increased (Kumar et al. 2011). Heavy metals are toxic, non-degradable contaminants, which can accumulate in living organisms (Fu and Wang 2011). Metal contamination can arise from both natural sources and human activities (Hurd et al. 2014). Estuaries are areas where contaminants accumulate (Guisti 2001), mainly because these are attractive places for human settlement. The Derwent Estuary in Tasmania, Australia, is heavily metal polluted, largely as a result of the legacy of past industry. The estuary has been monitored for more than 15 years (Whitehead et al. 2010; Coughanowr et al. 2015), and this clearly shows that, despite the marked reductions in metal inputs, some areas remain highly contaminated, particularly in the middle estuary, whilst the lower estuary has much lower levels (Whitehead et al. 2010).

Where metal pollution is a concern there is the potential for bioaccumulation, and this can have adverse implications for both the ecosystem and human health. Biomonitoring is a technique that provides information on the contaminants that may affect ecosystems (Phillips and Rainbow 1994; Zhou et al. 2008). There are two ways that this can be achieved, the first is the 'active' approach that relies on native organisms to monitor environmental conditions, the second is the 'passive' approach, which relies on transplanted organisms placed in the selected study area for a fixed period of time (Lacroix et al. 2015; Ronci et al. 2016). Active biomonitoring (ABM) is the approach most commonly used to evaluate metal impacts in the environment and is the focus of

most environmental monitoring programs. However, a combination of the ABM and passive biomonitoring (PBM) can provide a better understanding of the impacts on the broader ecology (Brown et al. 2012) as this approach would provide an appreciation of both the short-term (PMB) and longer-term impacts (ABM). Where pollution exists it may be desirable to find a way to remediate the affected areas and reduce detrimental impacts to the aquatic environment. Bioremediation is a process that utilises living organisms, which have the ability to accumulate or degrade chemical contaminants into less toxic forms in order to clean up polluted environments (Kumar et al. 2011; Juwarkar et al. 2010; Vidali 2001). Heavy metal removal is a cost-effective alternative where bioremediation has been demonstrated in a range of organisms such as bacteria, fungi, plants and algae (Juwarkar et al. 2010; Vieria and Volesky 2000).

Many seaweed species (Phyla Ochrophyta (brown), Rhodophyta (red) and Chlorophyta (green)), have been shown to have the potential to accumulate metals in ABM (Ryan et al. 2012; Boubonari et al. 2008; Zbikowski et al. 2007; Brown et al. 1999; Ho 1990; Luoma et al. 1982), but there is little information on the potential of algae to be used for PMB.

There has been a number of studies looking at the types of seaweeds that would be most suitable for bioremediation of nutrients-enrichment, with many of these focussed on the interactions of aquaculture and Integrated Multi-trophic Aquaculture (IMTA) (Buschmann et al. 2009; Zhou et al. 2006; Chopin et al. 1999; Troell et al. 1999; Troell et al. 1997; Petrell and Alie 1996), but there is less information about the specific applications of seaweeds for bioremediation of metal polluted environments.

Some studies have evaluated heavy metal effects in different species of seaweed, looking at physiological responses and ultrastructural alterations under laboratory or controlled conditions (Baumann et al. 2009; Felix et al. 2014; Gouveia et al. 2013; Zakeri and Abu Bakar 2012; Pellegrini et al. 1991; Santos et al. 2014). However, although laboratory experiments can provide useful information, there is still a need to understand how such species might perform under real world, field conditions. Field experimentation, particularly information obtained from transplant experiments, should complement laboratory experiments (Rainbow 1995), and can provide a much better overall understanding of bioaccumulation and remediation potential. Although there are some studies that have looked at *in situ* metal uptake or metal release in seaweeds (Eide and Myklestad 1980; Ryan et al. 2012; Malea et al. 1995; Boubonari et al. 2008; Ho 1990; Myklestad and Eide 1978; Ho 1984), other have examined *in vitro* how heavy metal pollution affects the physiological performance and ultrastructure of seaweeds species such as *Ulva* sp., *U. laetevirens* and *U. intestinalis* (Schiavon et al. 2012; Vecchia et al. 2012) and *U. intestinalis* (Andrade et al. 2004). A field study of *Ulva lactuca* and *Sargassum stenophyllum* found them to be relatively pollution-tolerant, but showed that in relation to an increasing urban pollution gradient the physiological performance of *S. stenophyllum* declined while *U. lactuca* improved along an urban pollution gradient (Scherner et al. 2012). In general, the cosmopolitan Ulvacean species are good indicators of pollution (Rainbow and Phillips 1993). Selected *Ulva australis* for the focus of this study as recent research has shown that it takes up metals and is widely distributed throughout of the Derwent Estuary (see above).

There is evidence that *Ulva* can tolerate metal pollution and that under controlled conditions it may be useful for metal remediation, but it is still not clear how effective

these algae might be as *in situ* remediation tools in highly metal polluted systems. Under highly polluted conditions, the remediation potential of seaweeds may actually be limited by their ability to survive the complex mix of anthropogenic stressors that are present at these locations (Pinto et al. 2003; Volesky 1990). In this study, we assess *in situ* the biomonitoring and bioaccumulation potential of *Ulva australis* to determine whether it might be a suitable management tool in a highly metal-impacted environment. The aims of this study were to use deployed thalli to evaluate whether metal uptake compromises the physiology of *U. australis*, to determine to what extent *U. australis* can accumulates metals in contaminated areas, and assess the management implications for using *U. australis* as a biomonitor.

6.3 METHODS

- **Study sites**

The study sites were located to reflect a range of metal impacted levels. An unpolluted control site was located 73 km southeast of Hobart city (42°52’ S: 147°19’ E) on Bruny Island (BI), well beyond the influence of any metal contamination from the Derwent Estuary (Fig. 6.1, Table 6.1). Two sites with historical “metal pollution” were selected from the Derwent Estuary (Coughanowr et al. 2015), to receive transplanted seaweeds collected from control site. Transplant site 1 (TS1, Lower Sandy Bay) is a recreational area located in the middle-lower estuary and transplant site 2 (TS2, Prince of Wales Bay) is an enclosed bay, situated in the middle estuary (Fig. 6.1). TS2 is mainly affected by contaminants derived from urbanisation, recreational activities and industrial discharge, and is characterised by high levels of metal such as cadmium (Cd), copper (Cu), lead (Pb) and zinc (Zn) identified in seawater and sediment. This region also has the highest levels

of ammonia+ammonium and nitrate-nitrite in the estuary (Coughanowr et al. 2015; Whitehead et al. 2010).

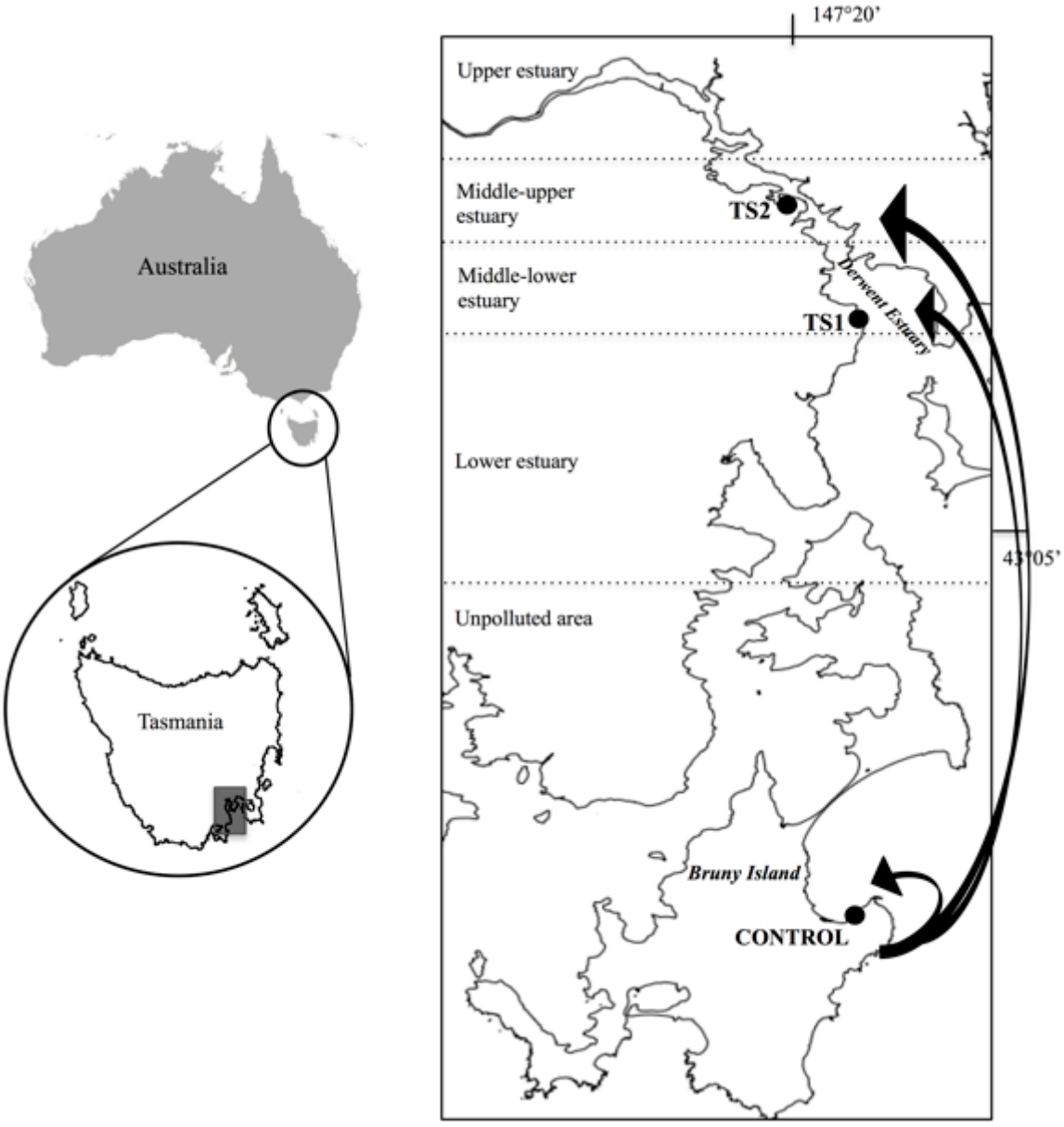


Figure 6.1. Study area indicating control and experimental sites, transplanted site 1 (TS1) and transplanted site (TS2) in the Derwent estuary, Tasmania.

Table 6.1. Environmental conditions for study sites and metal content (mg·kg^{-1}) in *Ulva australis*. Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2).

Parameter/Site	**Control**		**TS1**		**TS2**	
	Experiment	FRDC *	Experiment	DEP 2015**	Experiment	DEP 2015**
Salinity	34.9 ± 0.1		33.1	30 ± 2	26	19 ± 4
Temperature (T°C)	15 ± 0.1		15.5	14 ± 2	14.8	14 ± 2
O_2 (mg·L^{-1})	9.08		7.9	8 ± 2	7.4	9 ± 2
Ammonia/Ammonium (μg·L^{-1})		0.59 ± 0.3		15	57	74
Nitrite/Nitrate (μg·L^{-1})		1.50 ± 0.3		15	43.8	50
Nitrogen (μg·L^{-1})				280		360
Total Phosphorus (μg·L^{-1})				40		56
Dissolve Phosphate		0.23 ± 0.1				
Total Zn in surface water (μg·L^{-1})	3.16 ± 0.8			12 ± 2	28.2	21 ± 2

Parameter/Site	**TS2**			
	t_0	t_1**	t_2	t_3**
Salinity		26.78 ± 0.4		29.6 ± 1.2
Temperature (T °C)	20.1 ± 0.6	20.7 ± 0.1	20.5 ± 0.1	21.8 ± 0.2
Light (μmol·m^{-2}·s^{-1})	271.9 ± 0.1	105 ± 22.8	88 ± 20.4	216 ± 90.6
O_2 (mg·L^{-1})		8.44 ± 0.04		7.03 ± 0.26
Total Zn in surface water (μg·L^{-1})	20	21	24.5	31
Zn in *Ulva australis* (mg·kg^{-1})	18.8 ± 2.4	217 ± 38.8	485 ± 73.1	731 ± 110.5

* Data courtesy of Storm Bay project, FRDC project 2014/031. Values expressed as dissolved concentrations.

** Data courtesy of Derwent Estuary Program, DEP. Values expressed as total concentrations.

6.3.1 Metal content in *U. australis* and physiological and ultrastructural performance.

To evaluate metal accumulation capacity and whether metals can compromise *U. australis* performance, I used a passive biomonitoring approach to analyse physiological and ultrastructural responses.

Thalli were collected from the control site, on 19th August 2015 and transplanted back into the same site, and to sites TS1 and TS2. Forty-five individuals of 3.1 ± 1.5 grams were haphazardly collected from the shoreline at low tide and transported in an insulated container with seawater to be transplanted elsewhere at the control site, and at TS1 and TS2 the same day.

Ulva thalli were weighed and placed in each of five plastic mesh (0.6 cm mesh pore) baskets that were 18 cm height x 13 cm diameter (n = 5). Each of the five baskets was attached to a 1.5 m long x 2.5 cm diameter steel pole: the baskets containing *Ulva* plus the pole comprised one experimental unit (Fig. 2). Three experimental units (n = 3) were pushed into the sediment by snorkelling ensuring the top of the baskets were deployed at 2 m depth at high tide. The poles at each site were positioned to ensure they were 5 m away from one another. Limited replication (n = 3) was due to practical deployment conditions on the field. After 12 days, the *Ulva* thalli at each site were collected and metal content, growth rate, photosynthetic performance and concentration of photosynthetic pigments were assessed (see below). The ultrastructure of each thallus was examined (see below) and any differences noted. Additionally, 'ambient' *Ulva* plants (i.e. those that had grown naturally at each site) were collected from Control site, TS1 and TS2, from the shoreline during low tide for metal comparison with the metal content in transplanted thalli.

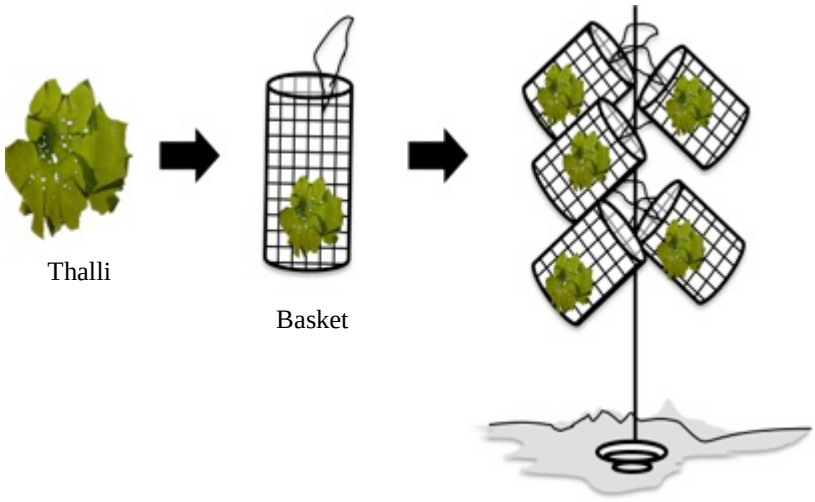


Figure 6.2. Schematic diagram of experimental design for transplanting thalli of *Ulva australis* for *in situ* assessment at the study sites.

- **Metal content in thalli**

Metal content in *U. australis* was measured at the start and end of the experiment on both the transplanted and ambient thalli. *Ulva* samples were transported to the laboratory in an insulated container, then rinsed to remove epiphytes and sand following the methods of Gledhill et al. (1998). To avoid contamination, all plastic ware were washed with alkaline detergent Decon 90® 2 % solution, and acid bath 5 % HNO_3 for at least 24 h. All material was then rinsed with Milli-Q® water (18 MΩ).

Initial wet weight (Wi) was recorded prior to freezing at -24 °C for up to a week until analysis. Frozen samples were then freeze-dried (FreeZone 4.5 Labconco) for 48 h until constant weight and the final weight (Wf) recorded. Samples were then ground in a coffee grinder to <2 mm particle size. Ground freeze-dried samples were sent to Analytical Services Tasmania (AST) a NATA (National Association of Testing Authorities, Australia) certified laboratory, for analysis of As, Cu, Pb and Zn using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Laboratory certified reference material (CRM), dogfish muscle CRM DORM-2, was used to test the accuracy and precision of the analysis. The metal detection limit of this analysis was 0.1 $mg \cdot kg^{-1}$

Dry Matter Basis (DMB) for As, Cu, Pb and Zn. Any evidence of damage to the thalli was also noted as a potential response to the experiment.

- **Growth rate**

Growth rate (Gr) of *U. australis* was calculated by comparing the initial and final wet weight, following Yong et al. (2013). Gr (% day^{-1}) =$((Wt/Wi)^{1/t} - 1)*100$, where; Wt = Final wet weight (g), Wi = Initial wet weight (g) and t = time (days).

- **Photosynthetic performance**

Chlorophyll *a* fluorescence in *U. australis* was measured using a Pulse-Amplitude Modulated (PAM) Diving fluorometer (Walz, Effeltrich, Germany). PAM was configured at Gain 4 according to Scherner et al. (2013). Before every pulse measurement, seaweeds were dark-adapted for 20 min using Walz underwater clips. Measurements were performed *in situ* on each replicate (n = 3) transplant sample before the experiment was started and at the end of the experiment, and on the ambient *Ulva* thalli from every study site (n = 3). Photosynthetic parameters measured were maximum quantum yield of photosystem II (PSII) = Fv/Fm and maximum electron transport rate (ETR_{max}), which is the maximal relative rate of electron transport activity (μmol·electrons·m^{-2}·s^{-1}) (Schreiber et al. 2011; Longstaff et al. 2002) ETR_{max} was calculated from the rapid light curves (RLC) as described in Ralph and Gademann (2005) using the exponential curve model of Platt et al. (1980). All parameters were calculated by the PAM processor version 1.0 (PAM-Processor 2015) using R Core Team (2013).

- **Photosynthetic pigments**

Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) contents were analysed by collecting 0.5 g of *Ulva* thalli at the start and end of the experiment. Samples were frozen at -20 °C until analysis. For pigment extraction, thalli were placed in 15 mL polypropylene tube (NEST®) with 3 mL dimethyl sulfoxide 99.9% (DMSO Sigma-Aldrich) for 1 h in an oven at 40 °C, then 200 µL was added to a 6.4 mm diameter 96 wells microplate (IWAKI) and absorbance was read using a micro-plate reader (Biotek synergy™ HT) to detect Chl *a* (663 nm) and Chl *b* (645 nm). Pigments were quantified according to Wellburn (1994) and the results of photosynthetic pigments expressed in $\mu g \cdot g^{-1}$ wet weight.

- **Ultrastructure**

Samples for ultrastructure analysis were collected at the end of the transplant experiment. Individual *Ulva* were sampled to collect 1 mm length sections. These were placed in a vial and fixed in 2 mL 2.5% glutaraldehyde, 0.05 M sodium cacodylate buffer (pH = 7.2) and 2% paraformaldehyde solution overnight. Samples were post-fixed with 1% osmium tetroxide and 0.05 M sodium cacodylate for 8 h followed by dehydration in an ethanol gradient. Fixation time and reagent concentrations were modified from those previously described by Bouzon et al. (2011). Finally, samples were embedded in Spurr's resin (Low viscosity, ProSciTech) to produce a solid block, which was then cut with an ultramicrotome (Leica EM UC7) at 60 nm before viewing in a Transmission Electron Microscope (JEOL JEM-1011 TEM). Block cutting and TEM analysis were undertaken at the Central Laboratory of Electron Microscopy, Federal University of Santa Catarina, Florianópolis, Brasil.

- **Environmental data**

The Derwent Estuary Program (DEP) provided details on the total Zn ($\mu g \cdot L^{-1}$) and nutrient (ammonium, nitrate and total phosphorus) concentrations in surface seawater (0.1 m depth) at TS1 and TS2 at the time of sampling. In addition, surface seawater samples, at 0.1 m depth (250 mL), were collected for total metal analysis from the control site at the start of the experiment (Table 6.1), and nutrient data (ammonium, nitrate and phosphate) were provided by the Storm Bay project, FRDC project 2014/031 for the control site. Total Zn metal analysis and nutrients in water were analysed by Analytical Services Tasmania (AST). Water detection limits for Zn was 1 $\mu g \cdot L^{-1}$.

6.3.2 Bioaccumulation assessment

To address the bioaccumulation capacity of *U. australis* I conducted an *in situ* experiment in a highly metal-polluted site. The metal accumulation capacity in a long-term deployment and bioaccumulation rate were assessed as follows.

To determine metal uptake rate and metal bioaccumulation, individual thalli of *U. australis* (14.5 ± 1.2 g) were deployed on 6th January 2016 in individual baskets as described above. In this case, four mesh baskets each containing one weighted *Ulva* individual were attached to each pole, giving 6 experimental units, which were deployed at TS2. Samples were monitored for growth and metal accumulation (see above) at the start of the experiment (t_0), and at regular intervals (t_1 = 15 days, t_2 = 30 days and t_3 = 45 days) thereafter, with one basket being removed from each experimental unit by snorkelling at each time. Any evidence of damage to the plants was also noted as a potentially response to the treatment.

To evaluate the association between metal accumulated in thalli and metals available in the environment, surface water samples were collected with a 50 mL syringe and a filtered through a Sartorius Stedim 0.45 µm (Minisart®), into 50 mL plastic centrifuge tubes, and kept at -24 °C until analysis. Seawater samples were analysed by Analytical Services Tasmania (AST) using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Metals concentrations in seawater are expressed as $\mu g \cdot L^{-1}$. Data loggers (HOBO pendant®) were attached to three of the experimental units (n = 3) to provide details of any differences in the temperature (°C) and light ($\mu mol \cdot m^{-2} \cdot s^{-1}$) throughout the duration 45 days experiment.

Zinc bioaccumulation in *U. australis* is expressed as the Concentration Factor (CF), which is the ratio of the metal (Zn) content in seaweed (Co) and the metal concentration detected in seawater (Csw), CF = Co $mg \cdot kg^{-1}$/Csw $\mu g \cdot L^{-1}$ (Conti and Cecchetti 2003). Additionally, metal accumulation rate (Mr), provides an estimate of the metal (Zn) content in the thalli at experimental time (At_f) less the metal content in the thalli at the beginning (At_i) divided by time (t). $Mr = (At_f - At_i)/t$.

- **Statistical Analysis**

A one-way ANOVA (95% confidence interval) was conducted to evaluate differences in *Ulva* metal content, Gr, photosynthesis performance, and photosynthetic pigments content between transplanted thalli. Levene's test was conducted prior to analysis to assess normality and homogeneity of the data, no transformation were required. Analysis was carried out with SPSS IBM Statistic version 22.

Photosynthesis performance, changes in photosynthetic pigment contents, and changes in metal concentration of ambient versus transplanted plants were assessed using a two-way ANOVA (95% confidence interval, factors: site and treatment). Again, Levene's test was conducted to assess normality and homogeneity of variance, with variance heterogeneity was corrected using Weighted Least Square regression (WLS). A Tukey´s post-hoc test was conducted where significant differences were observed, in order to further clarify the difference between ambient and transplanted thalli. Analysis was conducted in R studio statistical software (R Core Team 2013).

The relationship between Zn accumulated in *U. australis* and total Zn in surface waters was assessed using a Pearson Correlation test, conducted in R studio statistical software (R Core Team 2013).

6.4 RESULTS

6.4.1 Metal content in *U. australis* and physiological and ultrastructural performance.

At the end of the 12 day experiment, As, Cu, Pb and Zn were detected in the transplanted *U. australis* at each of the three study sites, with Zn content being consistently higher than other metals (Fig. 6.3). However, As, Cu and Pb, were not accumulated to any significant level in *Ulva*, and so were not considered further in this analysis. For Zn, there were significant differences between control and transplanted sites ($p = 0.001$, $F = 11.908$, $df = 2$) with a strong interaction between site (contaminant level) and treatment (transplanted plants) ($p = 0.018$, $F = 12.148$, $df = 3$) (Fig. 6.4). Post-hoc comparison revealed significantly higher levels of Zn accumulated in thalli transplanted to TS2 compared to control plants ($p = 0.001$), Zn content was greater in transplanted thalli at TS2 than TS1 (p

= 0.001), and Zn was greater in transplanted thalli at TS1 in relation to the control (p = 0.015).

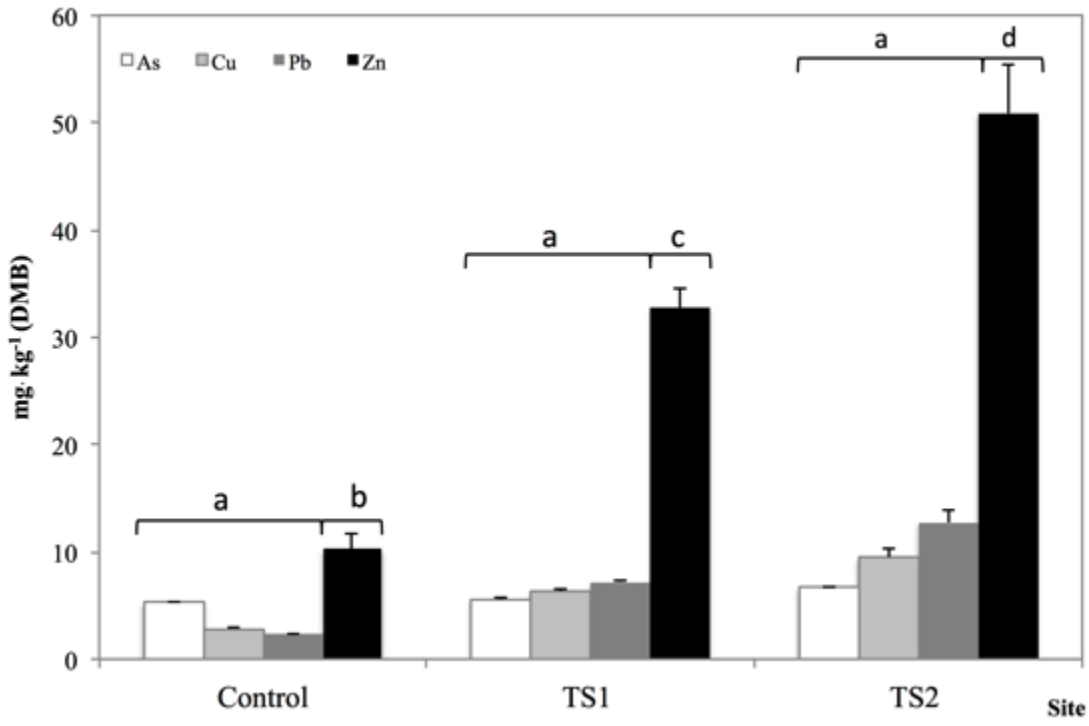


Figure 6.3. Metal content (mg kg^{-1} Dry Matter Biomass, DMB) in *Ulva australis*, Arsenic (As), Copper (Cu), Lead (Pb) and Zinc (Zn) after 12 days of transplantation. Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Values expressed on mean (± SE, *n* = 3). Letters above the bars indicate differences between Control, TS1 and TS2 shown as **a, b, c** and **d** (transplanted plants only). Same letters denoted no significant differences.

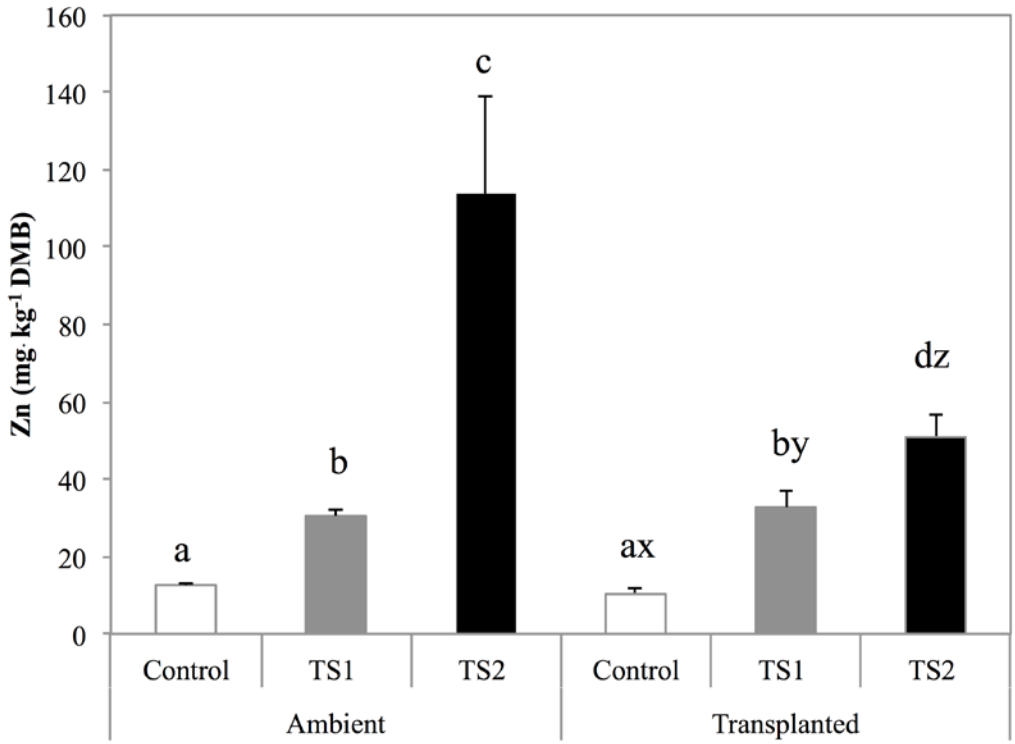


Figure 6.4. Zinc (Zn) content (mg kg^{-1} Dry Matter Biomass, DMB) in *Ulva australis* at Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Metal levels expressed as mean concentration (± SE, *n* = 3). Letters above the bars indicate how the transplanted thalli compare to ambient plants within each sites shown as **a**, **b, c** and **d**, and differences between Control, TS1 and TS2; shown as **x, y** and **z** (transplanted plants only). Same letters denoted no significant differences.

U. australis collected at the control sites had approximately 12.6 mg·kg^{-1} of Zn, compared with ambient plants that grown at TS1 and TS2, which had 30.5 mg·kg^{-1} and 113.7 mg·kg^{-1} DMB respectively (Fig. 6.4). After 12 days the levels of Zn in transplanted thalli at the control site were not markedly different ($p > 0.05$) from that observed in the control ambient plants (i.e. in 12 days plants accumulated 81.5% of the initial content or 10.3 mg·kg^{-1}). However, at TS1 the Zn content in the transplanted *U. australis* had increased to 107.3% of ambient levels or 32.8 mg·kg^{-1}. Interestingly, transplanted *Ulva* at the most polluted site (TS2) took up only 44.8 % of the ambient loads (50.9 mg·kg^{-1}) (Fig. 6.4).

Zn bioaccumulation was measurable over 12 days of deployment, and CF values were lower at control and semi-polluted sites (TS1) than at TS2 the more polluted site (Table 6.2). There was a high correlation between the total Zn concentration in surface seawater and Zn accumulated in *Ulva* (r = 0.87, p = 0.001, df = 7).

Table 6.2. Concentration factor (CF) for *Ulva australis* experiment 1 (*n* = 3) and experiment 2 (*n* = 6), and metal accumulation rate (Mr) for experiment 2. Values expressed as mean (±SE).

Physiological and ultrastructural assessment

Site	Treatment	CF
Control	Transplanted	5.2 ± 0.6
	Ambient	6.3 ± 0.1
TS1	Transplanted	5.5 ± 0.7
	Ambient	7.5 ± 1
TS2	Transplanted	5.1 ± 0.6
	Ambient	11.4 ± 2.5

Bioaccumulation assessment

		CF	MR (mg·kg/ day)
TS2	t0	0.9 ± 0.1	-
	t1	10.4 ± 1.8	13.3 ± 3
	t2	19.8 ± 3	17.9 ± 4
	t3	23.6 ± 3.3	24.3 ± 8

- **Physiological performance**

Growth rate, maximum quantum yield (Fv/Fm), the maximum electron transport rate (ETRmax) and photosynthetic pigments content were not affected by the different metal contents at the transplant locations. No significant differences (p >0.05) in growth rate, Fv/Fm, ETR_{max} (Table 6.3) or photosynthetic pigments (Chl *a* and Chl *b*) between control and transplanted thalli at either of the contaminated sites were observed (Table 6.3). Similarly, there was no significant difference (p >0.05) in growth rate, Fv/Fm, ETR_{max} (Table 6.3) or photosynthetic pigments when transplanted plants were compared with ambient plants (Table 6.3).

Table 6.3. Physiological and photosynthetic responses of *Ulva australis in situ* assessed after 12 days of transplantation. Growth rate (GR, % day^{-1}), Fv/Fm, ETR_{max} (µmol electrons·m^{-2}·s^{-1}) and photosynthetic pigment, Chl *a* and Chl *b* (µg·g^{-1}). Control site, transplanted site 1 (TS1) and transplanted site 2 (TS2). Values expressed as mean of measured parameter (± SE, *n* = 3).

Parameter	**GR (% day^{-1})**	**Fv/Fm**		**ETR_{max} (µmol·electrons·m^{-2}·s^{-1})**		**Chlorophyll *a* (µg·g^{-1})**		**Chlorophyll *b* (µg·g^{-1})**	
Site/Treatment	12 days	12 days	Ambient	12 days	Ambient	12 days	Ambient	12 days	Ambient
Control	4.6 ± 1.4	0.79 ± 0.03	0.79 ± 0.1	8.3 ± 0.2	12.3 ± 2.3	78.5 ± 3.3	71.6 ± 10.5	36.2 ± 1.7	33.9 ± 5.0
TS1	3.01 ± 1.2	0.83 ± 0.01	0.77 ± 0.06	14.6 ± 1.4	10.2 ± 0.1	105.7 ± 46.6	80.3 ± 6.2	65.1 ± 18.9	38.1 ± 3.7
TS2	6.1 ± 0.9	0.82 ± 0.02	0.86 ± 0.0	17.3 ± 2.9	20.8 ± 4.03	77.03 ± 7.9	61.8 ± 8.3	40.7 ± 7.1	32.6 ± 4.2

- **Ultrastructure**

The cytology of the control cells shows that the internal organelles were well organised, and the key cellular components, such as chloroplast, starch grains, lipid drops, Golgi body and vacuoles, are clearly visible (Fig. 6.5a). Chloroplasts are delimited by vacuoles and the thylakoids are parallel enclosing starch granules (Fig. 6.5b), a mitochondrion is visible on the chloroplast (Fig. 6.5b-c), the cell wall shows organised cellulose microfibrils sitting parallel with each other (Fig. 6.5c-d). A few mitochondria are visible associated with the chloroplast and Golgi (Fig. 6.5e).

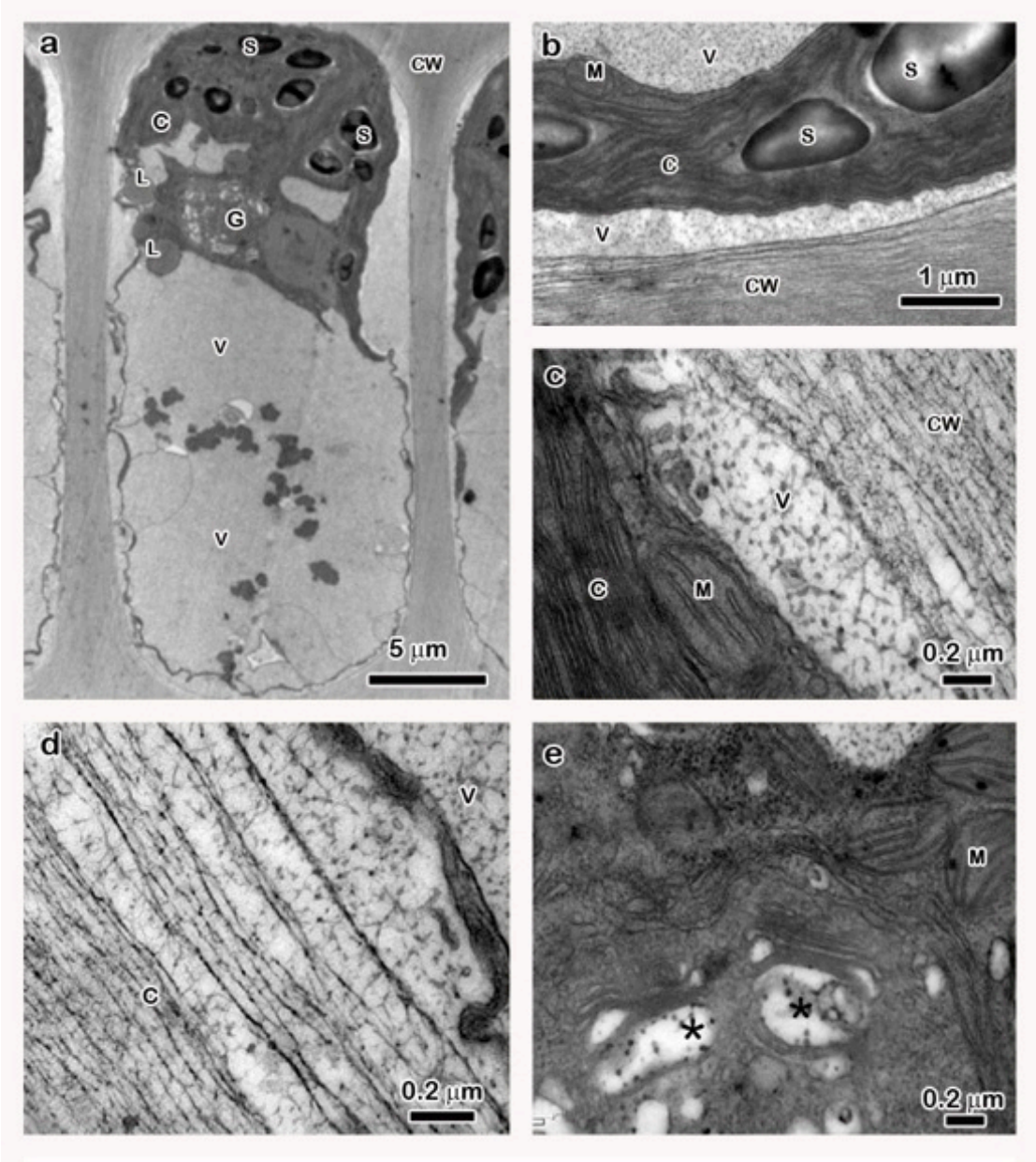


Figure 6.5. Transmission Electron Microscopy (TEM) micrographic images of *Ulva australis* ultrastructure from control thalli. A) cell general view with internal components well organised, b) cell wall, starch granules and chloroplast, c) cell wall, vacuole and thylakoids of chloroplast parallel organised, d) cellulose fibrils of cell wall and e) mitochondria on chloroplast. C: chloroplast, CW: cell wall, G: Golgi, L: lipids granules, M: mitochondrion, S: starch granules, V: vacuole and *internal Golgi material.

However, twelve days after being transplanted to a semi-polluted site (TS1), there were marked changes in the cell cytology. Cells showed evidence of electron-dense material in the vacuole, and an increase in the number of mitochondria in relation to the control (Fig.

6.6a, c and d). Thylakoids of the chloroplast appear to be organised in groups and slightly disarranged and enclosing a starch grain (Fig. 6.6b-c). Cellulose fibrils of the wall are irregularly positioned and electron-dense material deposited that was not observed in the control (Fig. 6.6c-d). Thalli transplanted to the high-polluted site (TS2) showed a higher level of damage within the cell. The cytoplasm is retracted and modified vacuoles show that metals has effected plasmolysis, and the amount of starch grains is also noticeably reduced (Fig. 6.7a) in relation to the control. In a number of cells, electron-dense material was observed inside the vacuoles (Fig. 6.7a-b), and similarly on the cell wall (Fig. 6.7c). Although the thylakoids were not strongly affected, an increased in mitochondria number suggests increased mitochondria activity (Fig. 6.7d).

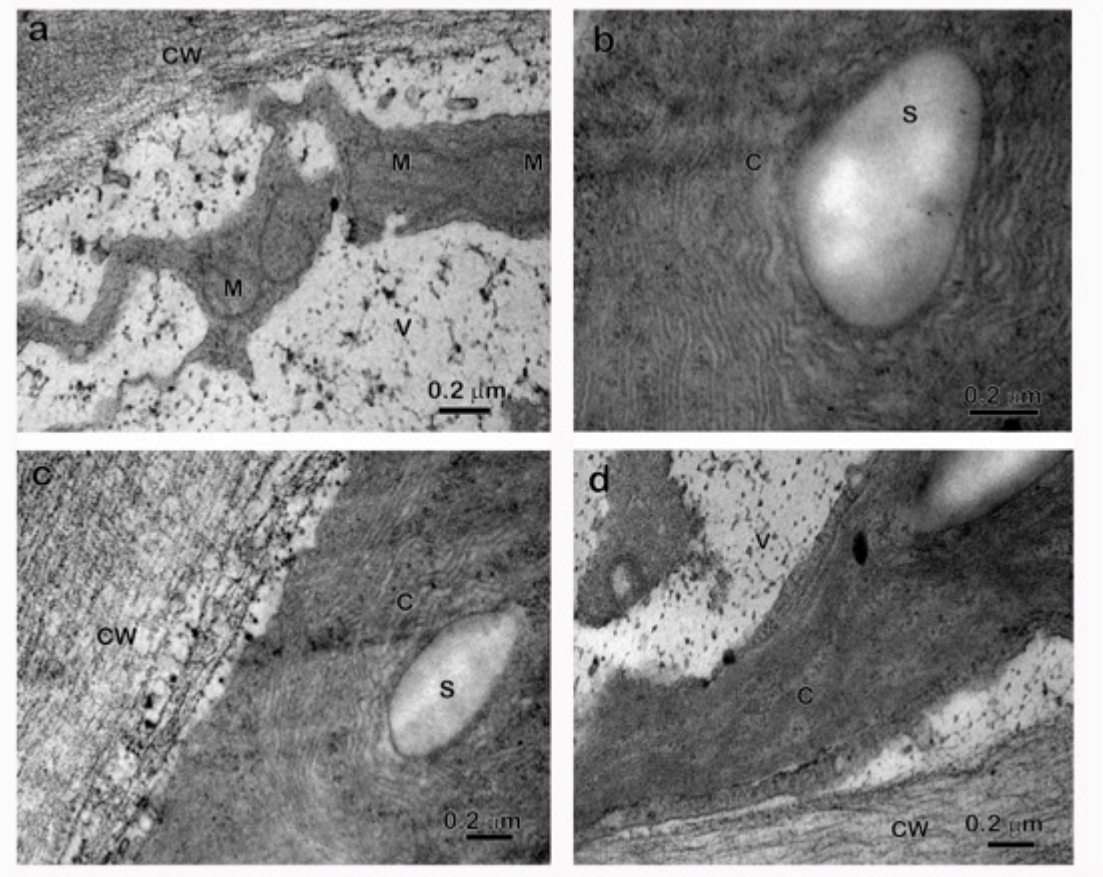


Figure 6.6. Transmission Electron Microscopy (TEM) micrographic images of *Ulva australis* ultrastructure in algae transplanted to a semi-polluted site (TS1). a) electron-dense bodies deposited on vacuole, b) thylakoids of the chloroplast slightly altered, c) electron-dense bodies deposited on

the cell wall, d) cell wall cellulose fibrils slightly unorganised. C: chloroplast, CW: cell wall, M: mitochondrion, S: starch granules, V: vacuole.

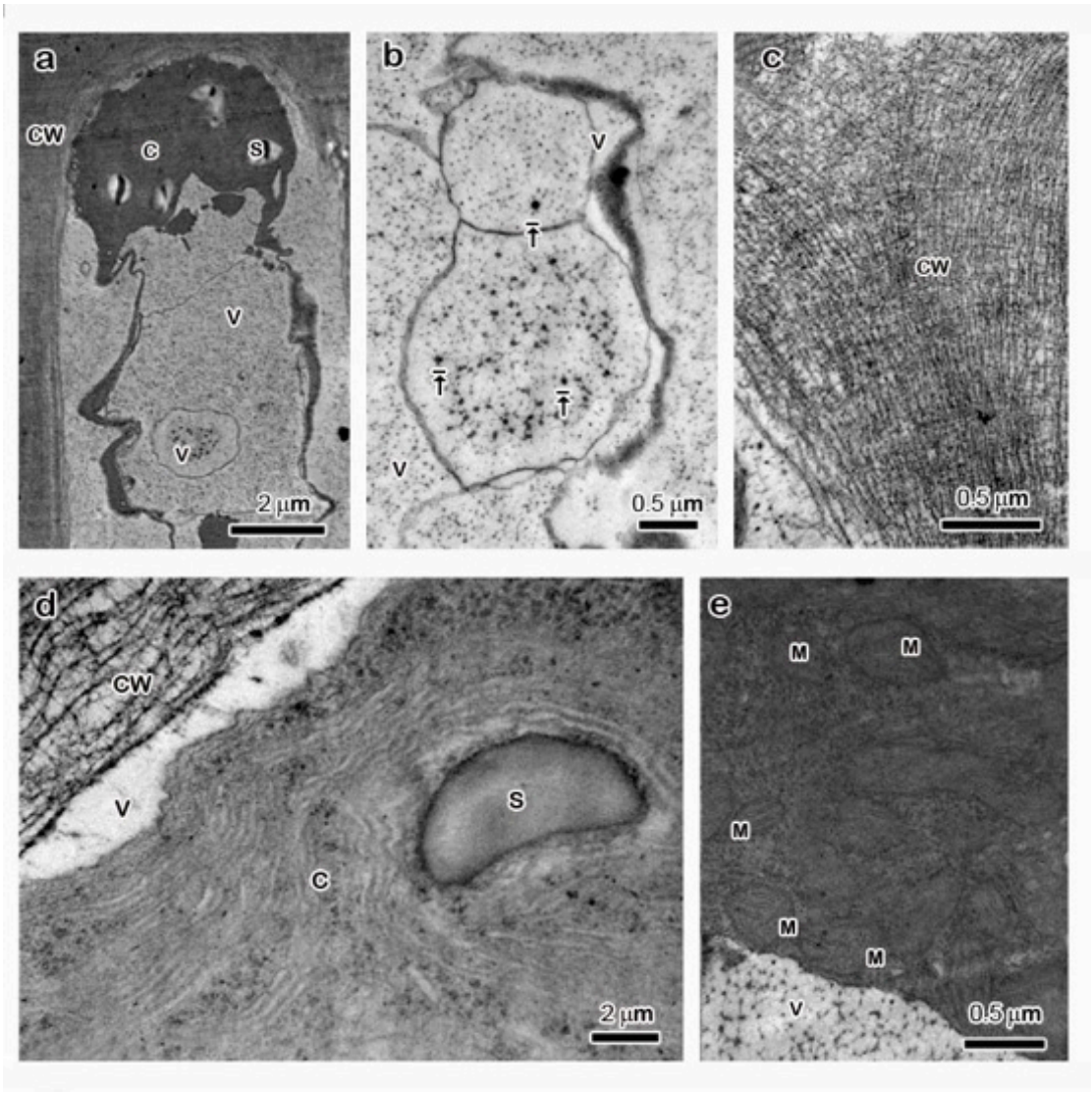


Figure 6.7. Transmission Electron Microscopy (TEM) micrographic images of *Ulva australis* ultrastructure transplanted to a high-polluted site (TS2). a) cell general view demonstrating irregularity on cytoplasm delimitation, b) in a number of cells electron-dense materials was observed inside vacuoles (arrows), c) cell wall fibrils and electron-dense bodies, d) chloroplast is not visibly affected and e) increased of mitochondria on chloroplast. Repetitive results were identified in all cell and samples analysed making the data robust. C: chloroplast, CW: cell wall, M: mitochondrion, S: starch granules, V: vacuole.

6.4.2 Bioaccumulation assessment.

There was clear evidence of an increase in Zn content in *U. australis* over time (Table 6.1), with a significant increase in the Zn content of the transplanted thalli, ($p = 0.001$, $F = 22.632$, $df = 3$). Although, there was no significant difference between Zn content in control and transplanted thalli after 15 days ($p > 0.05$) the levels were significantly greater at the transplanted sites after 30 days and levels continued to increase up to 45 days ($p = 0.001$). There was a strong correlation between total Zn concentration in seawater and Zn content in the *Ulva* ($r = 0.85$, $p = 0.001$, $dg = 22$). Bioaccumulation was evident after 15 days at the highly polluted site (TS2).

The Concentration Factor (CF) differed depending on length of exposure; increasing as metal exposure time increased. The metal accumulation rate (Mr) showed a similar pattern, also increasing over time (Table 6.2), from 13.3 $mg \cdot kg^{-1}$/day after the first 15 days, increasing to 17.9 $mg \cdot kg^{-1}$/day and 24,3 $mg \cdot kg^{-1}$/day after 30 days and 45 days, respectively. Zn content started at 18.8 ± 1.5 $mg \cdot kg^{-1}$ (t_0) and reached the highest concentrations, 863 $mg \cdot kg^{-1}$, in the transplanted thalli after 45 days (Table 6.1). The transplanted thalli appeared visibly degraded after 15 days, and the damage in the thalli continued to increase over the time, being markedly deteriorated after 30 and 45 days (Fig. 6.8).

Figure 6.8. *Ulva australis* thalli transplanted on the high-polluted site (TS2) to evaluated metal uptake rate. a) Initial thallus condition, b) transplanted thallus at 15 days, c) 30 days and d) 45 days.

6.5 DISCUSSION

This study provides evidence of metal accumulation in *U. australis*, confirming that *in situ* deployment of this species can be an effective means to monitor metal pollution, but also suggesting that *Ulva* deployment may have the potential to remove metals under certain conditions.

Passive biomonitoring (PBM) assessment showed that transplanted *U. australis* thalli accumulate metals to levels similar to those detected in ambient plants, with the final Zn content reflecting background contamination levels. Metal content in algae clearly increased as metal levels in the environment increased, which is consistent with previous observations (Mehta and Gaur 2005). In order to utilise seaweeds effectively as a bioindicator it is important to know how long it takes to achieve a level of contamination that would be equivalent to that observed in seaweed under ambient conditions. In this case, equilibrium with ambient metal content was achieved in a short period of time, 12

days. After 12 days, there was a linear correlation between the level of metals accumulated in the transplanted thalli and the metal available in the environment. It has been suggested that a linear relation between these parameters is a indication of a good bioindicator (Hurd et al. 2014; Rainbow and Phillips 1993). Long-term deployment, 45 days, showed that *U. australis* could accumulate Zn at very high concentrations, and that accumulation increased linearly over the time. The mean Zn concentration at 45 days (731.4 $mg \cdot kg^{-1}$) was 58 times higher than that observed at the control site. This was considerably higher than levels previously reported for *U. lactuca* of 66 $mg \cdot kg^{-1}$ from polluted sites (Ho 1990), and 10 $mg \cdot kg^{-1}$ in *Ulva* sp. from unpolluted areas (Ryan et al. 2012). These results are consistent with those previously reported for Zn in transplanted *Ascophyllum nodosum* and *Fucus vesiculosus,* where Ho (1984) in a seminal study found that Zn had increased 19% and 95% respectively, after two month compared to the concentration detected in ambient plants.

Zn content in *Ulva* assessed over 45 days was markedly higher than that in plants deployed for only 12 days, suggesting that a longer exposure time enhanced bioaccumulation. Bioaccumulation is the active process by which an organism takes up metals, in this process the organisms can resist/ tolerate particular toxicants (Oliveira et al. 2011). However, we observed that the longer the plants were deployed the greater thalli deterioration was observed. Whilst this could be just natural deterioration related to the seasonal life-cycle of *Ulva* (Hurd et al. 2014), it could also be related to experimental conditions (e.g. experimental baskets). Consequently, we would suggest limiting the deployment time for *U. australis* to no longer than 20 days, as this time-frame allowed plant to accumulate high concentrations of Zn (~ 300 $mg \cdot kg^{-1}$) without visible

deterioration. This strategy would enable the uptake of high amount of metals and quick "clean-up" of toxicants in the environment.

Metal accumulation was greatest at TS2, which is one of the most polluted areas, both in terms of metals and nutrients, in the Derwent Estuary (Coughanowr et al. 2015). Several studies have suggested that ambient concentrations of nutrients such as nitrate, ammonium and phosphate can influence metal accumulation (Lee and Wang 2001), with an increasing metal uptake capacity when nitrogen is widely available (Pinto et al. 2003). In a related species, *Ulva fasciata,* it has been shown that under controlled conditions the rate of Cd accumulation increased with increasing nitrate concentration (Lee and Wang 2001). Therefore, it might be suggested that high levels of nutrients (i.e. ammonium, nitrite and phosphate) and total inorganic nitrogen (360 $\mu g \cdot L^{-1}$) available at TS2 (Prince of Wales Bay) (Coughanowr et al. 2015), may have facilitated the metal uptake, providing the optimum environmental conditions for *U. australis* to accumulate metals.

Salinity has also been shown to affect metal uptake, increasing when salinity decreases. A previous study showed that metal concentration in *U. australis* sampled as a part of ABM was higher at less saline areas (D. Farias unpublished data). This may be a function of the fresh water having higher concentrations of metals than seawater (Hurd et al. 2014). The PBM in the current study showed that major ultrastructural changes were apparent at lower salinities. Cd absorption in the red algae *Pterocladiella capillacea* increase when salinity decrease resulting in damage to ultrastructure and negatively affecting physiological performance (Felix et al. 2014). Salinity has been recommended as a relevant parameter that should be considered when using seaweeds in estuarine biomonitoring programs (Mamboya et al. 2009; Munda 1984). The results suggest that the combined effects of low

salinity, high nutrients levels, and high metals loads at site TS2 would make this site an optimal location for biomonitoring, both ABM and PBM, as well as a good location for evaluating the *in situ* efficiency of *U. australis* as a potential bioremediation tool.

Internal cellular changes showed the effects of metal accumulation. Whilst, cellular changes were observed in transplanted *Ulva* plants from both sites, TS1 and TS2, there was a greater level of change (i.e. incorporation of electron-dense bodies and increase of mitochondria and vacuoles) in thalli from TS2 site. The presence of electron-dense bodies clearly indicates metal deposited within the cell, a feature previously observed in laboratory experiments (Chapter 4), and confirming the role of the cell wall in metal accumulation in *U. australis*. Previous laboratory experiments had demonstrated metal deposits in *Hypnea musciformis* exposure to Cd (Bouzon et al. 2011), in *P. capillacea* (Felix et al. 2014) and in *Ulva flexuosa* (formerly *Enteromorpha flexuosa*) exposed at 50 $\mu g \cdot L^{-1}$ Cu (Andrade et al. 2004). The increase in the number of vacuoles could be explained by the increase in Golgi activity. Golgi is an active organelle that form new vesicles (Lanubile et al. 1997), and cytoplasmic vesiculation has been related to the association between cell wall and metals ions in *Ulva laetevirens* exposed to Cd as a metal tolerance mechanism (Vecchia et al. 2012). This alteration has been observed in the microalga *Chlorella vulgaris* exposed to Cd, where it was also proposed to be a mechanism to improve metal-tolerance and maintain low cytosolic ions concentrations (El-Naggar and Sheikh 2014). There were more vacuoles observed in the transplanted thalli from TS2, suggesting that there was a higher level of cellular processes involved in managing metal accumulation. Interestingly, despite the marked changes observed in both metal accumulation and ultrastructure, there was no evidence of any significant effects on the growth rate, maximal photochemical yield (Fv/Fm), maximum electron transport rate

(ETR_{max}) or pigments content of transplanted *U. australis* thalli. This could suggest acclimation of transplanted plants.

Most of the investigations of the physiological effects of metal contamination on seaweed have been undertaken under controlled conditions. Unfortunately, the results of these lab-based experiments are highly variable and may not necessarily reflect the complexity of natural situation (Hurd et al. 2014). For instance, the concentrations of Zn and Cu can affect the Fv/Fm in Ulvacean (Baumann et al. 2009; Andrade et al. 2004; Han et al. 2008) and similarly, mercury (Hg) has been shown to severely affect the Fv/Fm of *Caulerpa racemosa, C. lentillifera* and *Ulva reticulata* (Zakeri and Abu Bakar 2012). While, Pb increased the electron transport rate ETR in *Sargassum cymosum,* Cu inhibited ETR after 7 days exposure (Costa et al. 2015). The current study was specifically developed to evaluate metal accumulation *in situ,* under the complex range of conditions that occur in nature, as demonstrated in *Padina gymnospora* and *Sargassum stenophyllum* (Amado Filho et al. 1999), and characterise the applicability of *U. australis* as a monitoring and management tool under field conditions. The results show that *U. australis* can be transplanted to metal polluted areas for a short period of time without affecting photosynthetic performance.

In a recent study, Zakeri and Abu Bakar (2012) suggested the maximum quantum yield (Fv/Fm) as the most effective parameter to evaluate metal toxicity in algae. Using this approach Scherner et al. (2012) showed *in situ* that Fv/Fm in *U. lactuca* increased 26 days after transplantation from clean to polluted areas, whilst Fv/Fm declined in the brown seaweed *S. stenophyllum* subject to the same conditions. This clearly demonstrated that there are differences in tolerance between species exposed to contamination. However, in this research, the photosynthetic performance is not the most relevant method to evaluate

in situ metal effects; following changes in ultrastructure is a valuable and novel tool that can clearly demonstrate metal accumulation effects in seaweeds, and one which would be add valuable in *in situ* assessment.

Whilst the results of this study clearly show the potential value of *U. australis* as a bioindicator of metal pollution and the effectiveness of this species in metal accumulation, the levels obtained at the more polluted sites do raise some concerns with respect to the potential for adverse environmental interactions. Concentrations of Zn accumulated in *U. australis* at the most polluted site (TS2) were high (above 700 $mg \cdot kg^{-1}$ after 45 days), and as such may provide a mechanism for transfer of metals into the food web (Young 1975; Huang et al. 2008). There are no specific guidelines for acceptable levels of metals in seaweeds, either in terms of seafood safety or environmental acceptability. However, it is suggested that levels of Zn in sediments should be below 410 $mg \cdot kg^{-1}$ to protect environmental values within aquatic ecosystems (ANZECC 2000). Whilst these values cannot be directly compared, the fact that the levels obtained in seaweeds are almost double that recommended for sediments might suggest that there is a risk here and further research might be needed to better characterize this before employing *Ulva* in the natural environment. The levels of Zn which are considered toxic for human consumption in crustaceans (<40 $mg \cdot kg^{-1}$), molluscs (130 - 290 $mg \cdot kg^{-1}$) and fish (<15 $mg \cdot kg^{-1}$) vary markedly (FSANZ 2006), but in all cases are considerably lower than those observed in *U. australis* at the most contaminated site after 45 days. As explained above, these levels cannot be directly compared and clearly *Ulva* used for remediation would never be used for human consumption. But the very high levels observed in *U. australis* at the most contaminated site might suggest that there could be an indirect risk of toxicity and/ or trophic accumulation.

6.6 CONCLUSIONS

Passive biomonitoring showed in 12 days that *U. australis* metal uptake rates reflected background loads, with higher uptake at the most polluted sites, showing the potential of this species as a bioindicator of metal pollution. Metal accumulation did not affect *U. australis* physiology as growth rate, photosynthetic performance (Fv/Fm and ETR_{max}), or photosynthetic pigments content. However, there was evidence of internal cellular changes (incorporation of electron-dense bodies) that could be related to metal load.

The bioaccumulation assessment, after 45 days, demonstrated that accumulation increased over time. However, there was deterioration of the thalli over time and as a result, it is suggested that 20 days is the most appropriate time for deployment *U. australis*. This timeframe would allow the plant to accumulate high levels of metals in a heavy metal impacted environment whilst remaining healthy and viable.

These recommendations will enable *U. australis*, to be more effectively used as a biomonitor to provide better understanding for managing metal pollution.

CHAPTER 7

General Discussion

Increasing population densities around estuarine and coastal areas leave these environments progressively vulnerable to metal pollution. Monitoring metal contamination in aquatic systems is necessary in order to identify sources of pollution, control contamination and to regulate impacts on ecosystems. Prior to undertaking this investigation, little was known about the tolerance, resistance or physiological responses of macrophytes to metal pollution within the study area or to what extent macrophytes could be used as indicators of ecosystem conditions ore even as a remediation tool. Therefore, this study provides new insight into the ecophysiological responses of macrophytes to metal pollution in one of the most metal polluted estuarine systems in the world, and suggests how macrophytes might actually improve our understanding and management of both the Derwent estuary specifically and estuaries in general.

The research presented in this thesis describes an objective approach for assessing the use of macrophytes for biomonitoring, and as a remediation tool in metal-impacted environments such as the Derwent Estuary (Hobart, Tasmania). By considering both biological and environmental factors, the research provides a better understanding of the dynamics of metal accumulation in macrophytes. The results demonstrated that macrophytes from the Derwent Estuary contained high content of metals (As, Cu, Pb and Zn) (Chapter 2). Zinc was the predominant metal contaminant in the estuary, present at high levels in both sediments and in the surface water, and was detected at high levels in the twelve macrophytes species assessed. However, only three species (*Ruppia megacarpa*, *Zostera muelleri* and *Ulva australis*) showed potential as bioindicators. The Zn content in

U. australis showed a clear spatial gradient, decreasing from the upper estuary to the mouth of the estuary, i.e. in a pattern similar to that observed in the concentration of surface water metals (Chapter 3).

Of the species evaluated, *U. australis* was found to be the best biomonitor, with potential for application throughout the Derwent estuary (Chapter 2 and 3). *U. australis* was found to have a high tolerance to metals and, as a result, laboratory and field trials were undertaken to assess its response under different Zn concentrations. The results showed changes in ultrastructure and evidence of metal accumulation at high metal concentrations. In order to demonstrate these effects it was necessary to develop a precise and targeted TEM technique for Ulvaceans (Chapter 4). Interestingly, although the results showed clear cellular changes with different metal concentrations, this did not cause mortality or affect the plant's physiology (Chapter 5 and 6). However, the cellular investigation did demonstrate the metal bioaccumulation capacity of *U. australis* by clearly showing the incorporation of electron-dense bodies in the cell wall, in the *in situ* ultrastructural assessment (Chapter 6). Overall, the results suggested that *U. australis* could be a useful tool to provide additional understanding of the biological uptake of metals in contaminated estuaries and thereby to improve management of these estuaries.

7.1 Evaluation of macrophytes as biomonitors of metal pollution

In chapter 2, it was observed that of all the macrophytes species evaluated (9 seaweeds and 3 seagrass) only three species (*U. australis*, *R. megacarpa* and *Z. muelleri*) had the potential to be used as bioindicators for metals. These species were shown to both take up metals and grow well in polluted areas. The two seagrasses species (*R. megacarpa* and *Z.*

muelleri) also showed promising results; they displayed high metal content and therefore it seems likely that they could be considered as bioindicators of metal pollution. Seagrass has been shown to be a useful indicator in previous studies (Govindasamy et al. 2011; Lafabrie et al. 2007). However, in the current study an important limitation to the use of seagrass as bioindicator is the fact that these species had a very restricted distribution; they are limited to very discrete areas within the Derwent estuary (Jordan et al. 2001), which means they would not be used to asses the whole estuary.

From the nine seaweeds species analysed, *U. australis* appeared to be the most suitable for use as a biomonitor. The species is sessile and has a wide distribution within the study area. It is a primary producer at the base of the food chain, and it can be readily sampled as it is an intertidal species, and it is able to accumulate a high level of metals (Chapter 2, 3 and 6). It is important when defining biomonitoring species that the technique for sampling be consistent at all study sites (Campbell 2002), the fact that *U. australis* was ubiquitous throughout the estuary allows this level of consistency. *Ulva* has a wide-tolerance to changes in temperature and salinity (Bird et al. 1998), and is distributed in the intertidal zone along the shoreline (i.e. from 0 - 5 m depth at low-tide level) (Edgar 2008), making it easy to sample/ assess, which in turn makes any changes in abundance (that could be related to environmental pressures) easily detectable. For all these reasons *U. australis* would appear to be a most suitable macrophyte species for biomonitoring in the Derwent Estuary.

7.2 Considerations for using *Ulva australis* as a biomonitor

Spatial and temporal assessments are necessary to evaluate the degree of contamination within a system, and are particularly important to distinguish anthropogenic and natural inputs (Villares et al. 2002) or specific changes (deterioration/ remediation) over time. Other Ulvacean species, *U. rigida* and *U. linza* (formerly *Enteromorpha linza*), have previously been used to demonstrate changes in metal pollution in aquatic systems (Haritonidis and Malea 1995). In the southern hemisphere, *U. lactuca* and *U. intestinalis* (formerly *Enteromorpha intestinalis*) were used very effectively to examine the levels of pollution in Otago harbour (Brown et al. 1999). Monitoring the Derwent estuary using *U. australis* over the two years of this study has clearly demonstrated the high levels of metal contamination in the system, with a strong spatial gradient in *Ulva* as well as evidence of temporal variability. These changes correlate well with the established environmental pollution gradient within the estuary (i.e. where the highest levels occur in the middle-upper estuary and decrease towards the mouth of the estuary) (Coughanowr et al. 2015). Spring and summer were identified as the best seasons for sampling, as this was when the plants accumulated the highest concentrations of metals (Chapter 3).

Ulva is an opportunistic genus and an important indicator of anthropogenic impacts; providing a reliable signal of both eutrophication (excess of nutrients) (Orfanidis et al. 2001) and metal pollution (Rainbow and Phillips 1993) that could create a niche for exploitation. In Australia, *U. australis* is a very common species, widely distributed on the rocky shores of the Australian coast (Edgar 2008; Kraft et al. 2010). However, this species is not restricted to Tasmanian waters and therefore it is application as a biomonitor can be expanded to other states of Australia, in fact, the genus *Ulva* is known as an bioindicator worldwide (Rainbow and Phillips 1993).

7.3 Algal physiology – implications for monitoring and assessment

Until this study, there was no reliable method for fixing green seaweeds for TEM scanning (Chapter 4). Effective fixation is critical to accurately assess metal contamination, as it allows direct comparison of the internal cellular components between specimens experiencing different levels of contamination (Shah et al. 2014). Poor cytological approaches can result in an inaccurate assessment of ultrastructure. Consequently, to ensure we fully understood the physiological responses of *Ulva* when exposed to metal pollution (Chapter 5 and 6), a new approach was developed to provide better fixation of the internal cell components. This new method can be applied to other Ulvacean species.

Using this approach the physiological and ultrastructural responses of *U. australis* to varying levels of metal pollution were investigated under both laboratory (Chapter 5) and field conditions (Chapter 6). Both showed that physiologically *U. australis* has a high tolerance to metal pollution; as there was no evidence of mortality and neither photosynthetic parameters i.e. Fv/Fm, and ETR_{max} nor photosynthetic pigments appeared to be affected at any of the metals concentrations, either under controlled or field conditions. Given this lack of physiological response, the presence of ultrastructure modifications (i.e. evidence of metal accumulation in the vacuole and the cell wall) was surprising.

Ultrastructural changes in response to metal pollution have been observed under laboratory conditions in a diverse range of red and brown seaweeds (Bouzon et al. 2011; Gouveia et al. 2013; Santos et al. 2015), and there have been a number of targeted laboratory studies of the effects of specific metals in *Ulva* species; with one study in particular clearly showing how the ultrastructure of *U. intestinalis* and *U. laetevirens* responds to cadmium

pollution (Vecchia et al. 2012). Other studies showed similar physiological responses with selenium in *Ulva* sp. (Schiavon et al. 2012) and cadmium in *U. lactuca* (Webster and Gadd 1996). However, fewer studies have been undertaken on the effect of metals in the field. Of those that have examined field responses Correa et al. (1999) showed a clear ecological impact as a result of copper contamination, and Leonardi and Vasquez (1999) noted changes in the ultrastructure of *Lessonia* spp. in the presence of copper. The *in situ* technique and associated assessment of the ultrastructural and photosynthetic response in *U. australis* outlined in this study represents a novel and more sensitive approach for evaluating metal bioaccumulation and remediation responses.

Both the biomonitoring and remediation potential of *U. australis* was clearly demonstrated by a relatively simple field experiment. *Ulva australis* thalli were translocated from a clean site (minimal metal exposure) to two sites with higher but different levels of metal contamination (one moderately contaminated and one highly contaminated). The results showed quite clearly that the translocated plants took up metals at a level that could be related to the site contamination level. These results showed that *Ulva* could not only provide a reliable indicator of current metal contamination levels but also highlight the potential to use deployed *Ulva* to reduce metal load. Whilst it is not clear just how much plant material or how long a deployment would be needed to make a difference, the results clearly demonstrated the potential. Having said this, further research will be necessary to clarify the optimal algal biomass for remediation and just how that might best be deployed. It is also important to note that there would be limitations with the current deployment design if it was to be scaled up i.e. it would be necessary to ensure that the deployment containers did not interfere with either light levels or water flow. It is essential to maintain a good light environment as this provides the energy for photosynthesis (Hurd et al. 2014),

whilst water flows determine nutrient availability and insufficient or excess flow will stress the plants (Hurd et al. 2014). Providing a suitable deployment technique would be fundamental for the success of any large-scale bioremediation deployments.

□□□ Recommendations for biomonitoring and management

Biomonitoring programs help in understanding contamination in estuaries and can be a useful tool for regulating impacts. However, it is important to ensure that the biomonitoring species selected are appropriate. The findings of this study identified a number of features (biological, practical and ecological) which suggest that *U. australis* would be a suitable biomonitor: it was able to provide information on metal contamination in a key functional ecological group within the Derwent (algae) and how that changed spatially and temporally. This suggests that *U. australis* would be a useful addition to the current routine monitoring program developed by the Derwent Estuary Program (DEP), complementing existing physical and chemical approaches.

The levels of metals observed in macrophytes from the Derwent were relatively high, and as such may indicate a potential trophic transfer risk, with the possibility of human health implications as a result of transfer of metals up the trophic chain (Chapter 1). Whilst in this case the risk from seaweed is really a second order issue, this does not mean it should be dismissed; where seaweed destined for human consumption has been shown to have high levels of metals levels this has resulted in immediate action, with toxicity levels for seaweeds becoming an important inclusion in European legislation (Besada et al. 2009). Although in this case, seaweeds are not a primary food product it may still be appropriate to recommend extending the investigation to look at the possibility of determining trigger values/risk level for metal contamination in macrophytes, and perhaps also consider how

this might be included in the Australian and New Zealand Environment and Conservation Council (ANZECC) guidelines as an indicator of broader ecosystem condition. Incorporating a risk level for metals in a cosmopolitan species such as *Ulva*, could provide a means to scale and compare pollutant levels globally (Rainbow 2006).

Macroalgae have a long history as reliable and efficient bioindicators of heavy metal pollution (Phillips 1977). As primary producers they are a key component of benthic communities and have been shown to be valuable indicators of the ecological status of benthic reef communities in coastal environments (Juanes et al. 2008; Orfanidis et al. 2001). The cumulative stress of anthropogenic impacts, such as heavy metal contamination, can affect both macroalgal and benthic communities, affecting reproduction and normal development (Orfanidis et al. 2003; Hurd et al. 2014). Further assessments of the effect of heavy metals on macroalgae (seaweeds and seagrass) from the Derwent Estuary are needed; as the high metal levels observed (Chapter 2) could negatively affect the ecological structure, assemblage, or recruitment patterns, and this may in turn affect the benthic foods webs.

Ulva australis was clearly shown to have value as a biomonitor and potential as a bioremediation tool in the Derwent estuary. This finding is consistent with the results for other *Ulva* species worldwide, for instance, *U. lactuca* was used to clean-up excess nutrients from integrated aquaculture systems in Saudi Arabia (Al-Hafedh et al. 2014); *U. ohnoi* has been used to remediate waste water outputs from aquaculture operations in western Australia (Lawton et al. 2013), and *U. fasciata* and *U. lactuca* have both been shown to be effective biosorption tools for metals in laboratory assessments (Ibrahim et al.

2016; Karthikeyan et al. 2007). However, although the biomonitoring potential of *U. australis* is clear we still do not fully understand its capacity for heavy metal remediation.

It has been suggested that seaweeds may not be effective for metal bioremediation because metals increase seaweed mortality (Bird et al. 1998). However, *U. australis* grows naturally throughout the Derwent Estuary, including in the most metal polluted regions. This demonstrates that *U. australis* is not only metal-tolerant (Chapter 5 and 6) but was also able to uptake metals to a high level, and therefore could potentially be used to remediate metal contaminated areas in the Derwent (Chapter 6). Since heavy metals are not readily degraded they will persist in the environment, and therefore active bioremediation is one of the few mechanisms that could be used to support recovery in metal impacted environments (Juwarkar et al. 2010). *Ulva australis* appears to be a good candidate for both assessment of metal pollution and bioremediation, and could even be used to assess the efficacy of bioremediation activities.

7.5 Conclusions

Overall, this study has shown that there is metal contamination in macrophytes species from the Derwent Estuary. Metals such as Zn, occur in high levels and could potentially be transferred to successive trophic levels (e.g. dietary exposure) or biomagnified, and this warrants further investigation given the potential impacts this might have on the broader environment, public health, and safety.

As a bioindicator of metal pollution, the differential uptake (tolerance) of metals shown by *U. australis* provides a valuable understanding of the spatial bioavailability of metals in the system. Although the component of this study looking at cellular changes did not provide a

clear diagnostic technique, it did identify a novel technique for *in situ* ecophysiological assessments, with *U. australis* being shown to be a valuable management tool for metal pollution in the Derwent Estuary.

Reviewing the application of *U. australis* in this study from a number of different perspectives - practical, physiological, temporal and environmental - shows its value as a suitable biotechnological tool for remediation with a high metal tolerance to heavy metal pollution and ability to uptake high levels of metals. In addition, this species could be used for the rehabilitation of impacted environments and minimise metal effects on the estuarine ecosystem.

7.6 Considerations for future research

A number of research limitations and suggestions for future research have been identified, but one of the key areas where further work is needed would be in seagrass ecology. Whilst the spatial survey in Chapter 1 identified seagrasses as a key indicator species in certain bays and in the upper region of the estuary, it was not possible to provide any further information on seagrass as a monitoring/ remediation tool. It is therefore suggested that future research be undertaken on the ecophysiology of these organisms. This would help to further explain the role of metals in vulnerable environments such as estuarine seagrass beds (Govindasamy et al. 2011; Ralph et al. 2006).

Another interesting extension to this research would be to determine whether macrophytes species can actually adapt to metal contamination, and whether some groups may be better at this than others as has been suggested (Stengel et al. 2004). Unfortunately, it was not

within the scope or timeframe of the current study to evaluate such potential for all of the groups of macrophytes investigated, but it would clearly be an interesting and logical extension of the current research. In Chapter 2, it was noted that species abundance and phylogenetic grouping differed considerably from the upper to the lower estuary. Whilst this variability could just be a function of changes in the natural environmental conditions, given the metal pollution history of the Derwent there may be some level of metal tolerance or adaptation factor associated with their distribution. Phylogenetic studies could be used to assess whether seaweeds are inherently adapted for metal accumulation or whether any adaptive capacity is an evolutionary process. This understanding could complement the assessment of the parameters discussed in this study, and help further improve our understanding of *Ulva*'s adaptation capabilities and its potential use as a bioindicator.

The current study showed some clear cellular changes (ultrastructure) but these did not appear to have any adverse effects on plants photosynthetic performance. One possible explanation is be that the observed cellular differences reflect a level of stress in the plants. Stress triggers oxidative damage to biological macromolecules such as enzymes and proteins (Hurd et al. 2014). Proteomics is increasingly being recognised as a useful technique for understanding environmental stress (Contreras and López-Cristoffanini 2012). Whilst this technique is still relatively new (Contreras and López-Cristoffanini 2012), early studies have shown that protein expression can be used to differentiate metal tolerance in brown seaweeds (Contreras et al. 2009). For instance, Zou et al. (2015) demonstrated that photosynthesis and energy metabolism proteins are reduced when *Sargassum fusiforme* is exposed to copper. Consequently, understanding protein expression and the physiological mechanisms involved in metal toxicity/ stress will

improve our understanding of the tolerance/ resistance of *U. australis* to environmental stress.

Chapter 6 clearly showed that *U. australis* had the potential to be a suitable remediation tool. This particular experiment was undertaken at a relatively limited spatial scale and as such further investigation at a larger-scale would be required in order to establish whether deployment of *Ulva* throughout the estuary would be feasible for bioremediation, and whether such an approach could be cost effective. If these additional assessments confirmed the initial assertion that *Ulva* proved to be a suitable approach for bioremediation, then the issue of how to dispose the contaminated *Ulva* would need to be investigated. The problem associated with the disposal of *Ulva* as a bioremediation product would depend on the particular pollutants accumulated (Bird et al. 1998), but in all the cases this is still an important consideration. Biofuel can be obtained from macroalgae, and *U. lactuca*, a species similar to *U. australis*, has been used to produce bioenergy as a secondary product (Bruhn et al. 2011); this may be one way to dispose of the contaminated biomass generated via metal bioremediation.

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